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Comparison of Extraction and Clean-up Methods Modified in Different Ways for the Determination of Pesticide Residues in Bees

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Abstract

As a result of the unconscious use of pesticides, honeybees are adversely affected, thus causing colony losses all over the world. In this research, different extraction and clean-up methods were compared on bee samples based on the QuEChERS method which is the most widely used pesticide analysis method in food. This research includes application studies for the multi residue method (MRM), which allows analysis of more than one pesticide. During the extraction phase, 7 different methods were compared, while the method with the most suitable repeatability and recovery values among these methods was taken to the clean-up phase. In the clean-up phase, 5 different clean-up methods were tried on the method which yielded the best values in extraction. In terms of average percentage recovery values, the 1st extraction method ranks first with 72 active substances between 70-120%, and the 3rd clean-up method with 92 active substances. Thus, while the number of active substances with suitable recovery values in the selected extraction method was 72, it increased to 92 with the continuation of the clean-up method, achieving a success rate of 92%. It is recommended to use this method in pesticide residue analyzes in bees with its outstanding features such as completing the analyzes in a short time, working easily with the infrastructure that can be found in all control laboratories, low analysis costs, ease of use, and reliability.

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1. INTRODUCTION

In the present day, pesticides are indispensable products used to fight against diseases and pests in crop production. Millions of tons of pesticides are produced and released into the environment each year. For this reason, pesticides are one of the most common and dangerous chemicals for non-target human, animal, and environmental health. The main usage area of pesticides is agricultural control. Biocidal products which are used to control pests and vectors on humans and pets are also a source of exposure to non-target organisms (Yavuz and Aksoy, 2016). During the spray application of pesticides, some amount of them evaporated and dispersed into the air while the remaining amount reaches the plant and soil surfaces. The evaporated pesticides are carried to other regions by the winds and then they may return to the earth with rain, fog, or snowfall. In this way, pesticides reaching other non-target organisms may cause residue and toxicity (Akdogan *et al*., 2012). Usage of pesticides, industrial agricultural practices, honeybee pathogens and global climate changes are among the activities that threaten the life of honeybees. Especially since the beginning of the 1990s, the use of pesticides has increased rapidly due to population growth and the need for agricultural products. The increase in pesticide use is one of the factors that negatively affect the lives of honeybees.

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As a result of the reckless use of pesticides, the negative effects of honey bees on their lives cause colony losses in the world. Pesticides used against agricultural pests not only affect honeybees flying over or pollinating the plants but also indirectly affect other individuals in the colony by carrying substances such as pollen and nectar containing pesticide residues to the hive (Polat *et al*., 2020). In a research, investigating the effects of neonicotinoid group pesticides on bees, it was determined that all the bees died within 24 hours after the pesticides with active ingredients Imidacloprid, Thiacloprid, and Thiamethoxam were used at the recommended dose for agricultural control; therefore it was determined that they were very harmful to bees. In this study, when acetamiprid was used at the recommended dose, 74% of the bees died within 24 hours. It has been determined that the survivors cannot use the necessary organs (Karahan *et al*., 2019). In another research, the effect of imidacloprid from the neonicotinoid group in winter loss on Anatolian honeybee (*Apis mellifera anatoliaca*) was investigated. In this research, 5 mL/100 L water and 6 different doses of this dose diluted 50% (2.5 mL/100 L water, 1.25 mL/100 L water, 0.625 mL/100 L water, 0.312 mL/100 L water, 0.156 mL/100 L water) was used. At the end of this research, 75.66% of the bees treated with different doses of imidacloprid died, while 49% of the control group bees died. In addition, it was observed that the bees treated with imidacloprid suffered more winter loss than the control group bees (Karahan *et al*., 2018). Alaux *et al*. (2010) conducted, exposed honeybees to imidacloprid, one of the neonicotinoid group pesticides and the nosema parasite. Co-exposure has been shown to severely weaken honeybees. Thus, it is thought that pesticides and parasites may have synergistic effects. Wu *et al*. (2012) found that bees growing on comb with high pesticide residue were infected with nosema parasite at a higher rate than those growing on comb with low residue. Kiljanek *et al*. (2016) carried out a method validation in which 200 pesticides can be screened with LC-MS/MS and GC-MS/MS with an extraction method they developed based on QuEChERS. They analysed more than 70 poisoned honeybee samples according to this method. The QuEChERS method is currently the most widely used sample preparation method for the analysis of pesticides by both research and control laboratories, especially in plant food samples, and it is reported that this method is used in 98% of scientific publications. In addition, articles on the use of complex matrices such as honey and pollen have also been published. In the coming years, it is predicted that it will be a predominantly preferred method by making small changes to improve the detection spectrum (Yavuz and Aksoy, 2016). In this research, different extraction and clean-up methods were compared on bee samples based on the QuEChERS method, which is the most widely used method in pesticide analysis in foods and application studies were carried out for the multi-residue method (MRM). Thus, it is aimed to be a pioneer in the validation studies for the MRM method, where hundreds of pesticides can be analysed together in bees.

2. MATERIAL and METHODS

2.1. Material

Since using as a blank sample in extraction and clean-up stages for the residues of some selected pesticides, it is very important that the bees to be used in the analyses do not contain any pesticide residues. For this reason, worker bees, which were destroyed at -18 °C, were supplied from BIOBEST, one of the important bumblebee production facilities in Antalya, to be used as blank samples in this research. The samples, which were transported to the laboratory in the cold chain, were kept in the deep freezer at -18 °C for the homogenization process. Afterward, the samples taken from the deep freezer were treated with dry ice at a temperature of -78.5 °C for another 3 hours and the samples were allowed to freeze thoroughly. Thus, a good homogenization (fragmentation) was achieved. After the blank samples were homogenized, the weighing phase started to be used in different extraction and clean-up methods. All certified pesticide standards supplied from Dr. Ehrenstorfer GmbH (Augsburg,

Germany) used in the analysis have a purity of over 90%. The chemicals used, such as water, methanol, ethyl acetate, n-hexane, formic acid, and ammonium formate, are HPLC grade and acquired from Merck KGaA (Darmstadt, Germany). The extraction and clean-up kits used belong to Agilent Technologies (California, USA). The device on which the analyses were made by Agilent Technologies (California, USA) 1260 Infinity LC, 6460 Triple Quadrupole LC/MS (Jetstream ESI) model.

2.2. Method

Methods have been modified in different ways based on the QuEChERS method, which is the most commonly used method for pesticide analysis in plant-based foods. While choosing the methods, the physiological characteristics of the bees' bodies (water and fat ratios) were taken into consideration. Therefore, solvents with different properties were tested due to the addition of water in different amounts in different weights and the body structure of the insects. During the extraction phase, 7 different methods were tried, and the method with the most suitable recovery values among these methods was taken to the clean-up phase. In the clean-up phase, 5 different clean-up methods were tried on the method with the best values in extraction.

2.2.1. Extraction Methods

Table 1. Extraction steps.

Extraction methods	1	2	3	4	5	6	7
Sample amount	10 g	5 g	10 g	5 g	10 g	5 g	15 g
Addition of water	2.5 mL	6 mL	2.5 mL	6 mL	2.5 mL	6 mL	3.75 mL
Addition of acetonitrile	10 mL	10 mL	7 mL	7 mL	7 mL	7 mL	15 mL (1% Acetic Acid)
Addition of ethyl acetate	-	-	3 mL	3 mL	-	-	-
Addition of N-hexane	-	-	-	-	3 mL	3 mL	-
Internal Standart (10 mg/kg Triphenyl Phosphate)	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL	75 µL
Pesticide mixture (1 mg/kg)	500 µL	250 µL	500 µL	250 µL	500 µL	250 µL	750 µL

Homogenized samples were weighed into 50 mL centrifuge tubes according to the values specified in Table 1, after adding water, they were shaken for 1 minute and held for 5 minutes. Then, solvents of the relevant method, internal standard and pesticide mixtures were added and shaken for 2 more minutes. In the first 6 extraction methods, after the shaking step, 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate and 0.5 g of disodium hydrogen citrate sesquihydrate buffer salts were added to the tubes. It was shaken for 1 min and centrifuged at 3000 rpm for 5 min. In the seventh extraction method, after shaking, 6 g of anhydrous magnesium sulfate and 1.5 g of sodium acetate were added to the tubes, shaken for 1 min and centrifuged at 1500 rcf for 1 min. After centrifugation, 1 mL was taken from the supernatant of the tubes to determine the recovery values. It was filtered with a 0.45 µm polytetrafluoroethylene (PTFE) filter and transferred to vials. 10 µl of 5% formic acid (prepared in acetonitrile) solution was added to the vials for injections in the LC-MS/MS.

2.2.2 Clean-up Methods

After centrifugation at the extraction stage, 6 mL of supernatant was taken into 15 mL tubes.

1. Clean-up method: The tubes were kept at -18°C for 4 hours so that the hydrocarbon chains in the fat and wax layer of the extract solidified and precipitated, and then centrifuged at 3000 rpm for 5 minutes.

2. Clean-up method: In order to purify the extract from organic acids, lipids and sugars, 900 mg magnesium sulfate and 150 mg primary secondary amine (PSA) were added to the tubes and shaken for 30 seconds.

3. Clean-up method: In order to purify the extract from organic acids, lipids and sugars, 900 mg magnesium sulfate, 150 mg PSA and 150 mg C18 sorbent were added to the tubes and shaken for 30 seconds.

4. Clean-up method: The tubes were kept at -18°C for 4 hours so that the hydrocarbon chains in the fat and wax layer of the extract solidified and precipitated, and then centrifuged at 3000 rpm for 5 minutes. Then, 900 mg magnesium sulfate and 150 mg PSA were added to purify the extract from organic acids, lipids, and sugars; then shaken for 30 seconds.

5. Clean-up method: The tubes were kept at -18°C for 4 hours so that the hydrocarbon chains in the fat and wax layer of the extract solidified and precipitated, and then centrifuged at 3000 rpm for 5 minutes. Then, 900 mg magnesium sulfate, 150 mg PSA and 150 mg C18 sorbent were added to purify the extract from organic acids, lipids, and sugars; then shaken for 30 seconds.

After shaking, the tubes were centrifuged at 3000 rpm for 5 minutes in all clean-up methods. Then, 1 mL was taken from the supernatant of the tubes to determine the recovery values. It was filtered with a 0.45 µm PTFE filter and transferred to vials. 10 µl of 5% formic acid (prepared in acetonitrile) solution was added to the vials for injections in the LC-MS/MS. The working conditions of LC-MS/MS are given in Table 2.

Table 2. Instrument parameters.

Parameters	
Column	Poroshell 120 SB-C18, 3.0x100mm, 2.7µm
Column temperature (°C)	35
Mobile Phase A	Water (5 mM ammonium formate, 0.1% formic acid)
Mobile Phase B	Methanol
Gas temperature (°C)	300
Gas flow (L/min)	10
Nebulizer (psi)	40
Sheath gas temperature (°C)	250
Sheath gas flow (L/min)	11
Binary pump flow (mL/min)	0.45
Binary pump timetable	
0.5 min	A: 70% B: 30%
2.5 min	A: 30% B: 70%
11.5 min	A: 5% B: 95%
13.9 min	A: 5% B: 95%
14 min	A: 70% B: 30%
16.5 min	A: 70% B: 30%

Calibration curves with six levels and two replications were prepared at 5, 10, 25, 50, 100 and 200 µg/kg concentrations for each active ingredient using the matrix-matched calibration method. Correlation coefficients of all pesticide active ingredients analyzed were calculated as $R^2 > 0.99$. Data calculations and results on the device were made using the built-in software named MassHunter.

3. RESULTS and DISCUSSION

Pesticide mixtures prepared for 100 selected pesticide active ingredients in LC-MS/MS device were spiked into blank samples. Recovery studies at 50 µg/kg level with five replications were performed for seven different extraction methods. Relative Standard Deviation (RSD) values of the measured values were calculated and it was checked whether these values met the ≤ 20% condition. The RSD values for all analyzed pesticides met the condition of ≤ 20% and the average percent recovery values of five repeated measurements are given in Table 3.

Table 3. Average recovery values were determined in extraction methods.

	Average Recovery in Extraction Methods (%)						
Pesticides	1	2	3	4	5	6	7
Acetamiprid	99	95	114	99	99	91	100
Ametoctradin	67	68	81	68	70	54	67
Amitraz	78	64	68	62	58	54	60
Azoxystrobin	117	107	127	101	103	99	105
Bifenthrin	74	63	70	56	51	55	54
Boscalid	72	79	96	85	81	83	72
Bupirimate	102	91	106	85	78	80	87
Buprofezin	90	82	96	79	68	68	84
Carbendazim	74	83	71	66	67	69	82
Chlorpyrifos	76	68	73	67	60	50	57
Chlorpyrifos Methyl	78	64	82	58	61	64	80
Clofentezine	84	66	46	45	57	67	49
Clothianidin	82	95	80	84	96	109	87
Cyantraniliprole	69	75	63	80	68	98	55
Cyazofamid	77	72	90	82	67	77	81
Cyflufenamid	91	111	112	100	101	113	104
Cyflumetofen	85	73	84	71	70	63	78
Cymoxanil	74	68	80	68	66	61	69
Cypermethrin	120	147	157	156	151	159	149
Cyprodinil	78	73	91	73	54	59	66
DEET	95	91	100	87	82	75	84
Deltamethrin	127	129	122	136	58	148	100
Diafenthiuron	132	131	139	125	110	101	135
Diazinon	98	75	90	66	75	72	83
Difenoconazole	122	171	164	177	184	176	189
Dimethoate	81	79	96	84	75	71	83
Dimethomorph	132	139	143	136	123	125	125
Dodine	74	93	90	63	90	64	77
E-Fenpyroximate	95	97	88	88	68	76	82

Emamectin Benzoate	72	67	76	69	64	65	65
Famoxadone	66	107	101	137	80	107	88
Fenamidone	83	82	102	110	94	96	82
Fenbutatin oxide	68	60	63	58	63	66	63
Fenitrothion	93	101	109	65	91	73	76
Fenpropathrin	131	141	143	147	121	135	129
Fenthion	100	113	115	101	101	90	95
Fenthion Oxon	90	85	98	75	79	68	76
Fenthion Oxon Sulfone	92	86	107	92	72	77	78
Fenthion Oxon Sulfoxide	91	83	48	50	77	76	87
Fenthion Sulfone	113	106	126	92	103	90	100
Fenthion Sulfoxide	100	97	107	91	90	89	86
Fipronil	140	149	158	158	155	174	167
Flonicamid	73	94	103	101	94	92	81
Flubendiamide	150	178	183	163	163	158	144
Fludioxonil	72	94	77	84	71	67	72
Flufenoxuron	110	120	145	182	133	122	113
Fluopicolide	79	84	87	84	72	79	76
Fluopyram	90	78	89	80	80	71	77
Flusilazole	62	72	86	102	73	63	63
Fluxapyroxad	95	88	103	87	91	87	84
Hexythiazox	68	70	74	88	66	65	67
Imazalil	78	71	87	74	68	68	67
Imidacloprid	78	134	72	70	122	129	101
Indoxacarb	96	135	136	124	136	130	144
Isopyrazam	35	22	32	20	28	22	28
Kresoxim Methyl	102	84	101	82	90	86	87
Lambda Cyhalothrin	127	145	130	133	144	121	123
Malaoxon	96	92	102	83	87	82	88
Malathion	100	82	98	72	80	75	91
Methoxyfenozide	81	79	92	74	75	72	77
Metrofenone	101	77	87	73	83	89	95
Novaluron	109	162	146	152	175	152	169
Omethoate	64	64	76	70	63	65	65
Penconazole	67	88	93	102	79	79	77
Permethrin I	68	42	42	45	57	41	46
Permethrin II	62	48	52	58	60	48	44
Phenthoate	112	98	112	98	92	75	96
Phosalone	127	128	124	103	113	100	123
Phosmet	127	127	127	118	106	113	108
Phosmet Oxon	81	82	88	78	78	76	78
Pirimicarb	79	80	95	79	72	74	76
Pirimicarb Desmethyl	78	73	48	49	70	67	72
Propargite	89	93	97	86	81	74	82
Propiconazole	124	167	161	157	151	157	154
Proquinazid	89	86	91	91	42	44	85

Pyraclostrobin	83	61	74	60	67	69	72
Pyridaben	118	133	149	124	110	103	123
Pyrimethanil	54	63	67	56	41	43	59
Pyriproxyfen	79	82	86	76	71	69	75
Spinetoram	78	64	75	66	65	60	69
Spinosyn A	80	65	73	57	72	65	68
Spinosyn D	75	52	63	53	53	50	55
Spirodiclofen	118	112	111	101	92	88	101
Spiromesifen	70	57	72	52	45	47	47
Spirotetramat	115	106	156	161	139	142	108
Spirotetramat Enol	96	116	118	166	145	153	108
Spirotetramat Enol Glucoside	54	79	49	41	73	64	76
Spirotetramat Ketohydroxy	156	157	142	162	146	152	150
Spirotetramat Monohydroxy	113	105	158	165	149	138	113
Sulfoxaflor	93	77	90	68	71	71	87
Tau Fluvalinate	159	102	101	91	95	111	155
Tebuconazole	96	146	155	171	129	126	119
Tebufenozide	82	76	91	74	80	65	82
Tebufenpyrad	91	105	115	125	103	102	99
Teflubenzuron	131	109	126	85	99	101	89
Thiabendazole	60	66	52	57	60	62	65
Thiacloprid	81	76	92	82	71	73	73
Thiamethoxam	108	113	112	106	100	101	108
Thiophanate Methyl	72	64	104	85	78	72	64
Trifloxystrobin	100	60	79	61	71	63	74

When the Table 3 is examined, the extraction methods have respectively 72, 60, 64, 51, 55, 46, and 61 active substances in the recovery range of 70-120%. In this case, the highest recovery was obtained with the 1st extraction method. For this reason, the clean-up stage was continued with the 1st extraction method. The selected extraction method was tested via five different clean-up methods with five repetitions of recovery studies at 50 µg/kg. RSD values of the measured values were calculated and it was checked whether these values met the ≤ 20% condition. The RSD values for all analyzed pesticides met the condition of ≤ 20% and the average percent recovery values of the five repeated measurements are given in Table 4.

Table 4. Average recovery values are determined in clean-up methods.

	Average Recovery in Clean-up Methods (%)				
Pesticides	1	2	3	4	5
Acetamiprid	119	96	98	123	112
Ametoctradin	92	84	82	82	85
Amitraz	60	50	82	58	80
Azoxystrobin	126	115	112	128	118
Bifenthrin	15	25	86	20	80
Boscalid	94	92	92	100	103
Bupirimate	112	94	95	104	103
Buprofezin	96	93	92	97	95
Carbendazim	94	67	78	91	84

Chlorpyrifos	73	68	71	75	76
Chlorpyrifos Methyl	67	66	88	84	96
Clofentezine	75	78	78	78	89
Clothianidin	105	81	92	114	115
Cyantraniliprole	59	56	68	74	68
Cyazofamid	91	51	80	76	82
Cyflufenamid	103	89	107	120	116
Cyflumetofen	93	93	90	84	92
Cymoxanil	78	64	114	94	83
Cypermethrin	160	191	110	174	161
Cyprodinil	84	72	81	83	86
DEET	106	93	91	105	102
Deltamethrin	180	163	108	200	144
Diafenthiuron	140	139	153	152	151
Diazinon	110	99	92	114	114
Difenoconazole	138	119	120	143	176
Dimethoate	92	79	75	91	87
Dimethomorph	150	128	116	152	156
Dodine	75	48	70	53	33
E-Fenpyroximate	95	101	95	99	96
Emamectin Benzoate	82	76	71	79	71
Famoxadone	60	64	73	92	78
Fenamidone	99	90	95	96	109
Fenbutatin oxide	64	63	72	58	54
Fenitrothion	111	60	88	100	124
Fenpropathrin	141	153	124	170	148
Fenthion	107	100	102	112	120
Fenthion Oxon	88	88	75	96	85
Fenthion Oxon Sulfone	93	61	82	86	94
Fenthion Oxon Sulfoxide	84	65	96	99	98
Fenthion Sulfone	97	83	100	123	82
Fenthion Sulfoxide	113	91	92	108	110
Fipronil	138	168	118	166	159
Flonicamid	88	65	76	92	101
Flubendiamide	150	99	137	138	128
Fludioxonil	72	91	72	91	99
Flufenoxuron	104	125	102	144	118
Fluopicolide	90	93	100	113	111
Fluopyram	97	86	94	99	98
Flusilazole	64	60	71	68	66
Fluxapyroxad	93	86	87	104	106
Hexythiazox	72	75	76	80	84
Imazalil	80	75	75	83	82
Imidacloprid	133	90	102	137	153
Indoxacarb	142	102	100	164	167
Isopyrazam	56	53	68	58	60
Kresoxim Methyl	119	102	87	111	112
Lambda Cyhalothrin	118	137	118	146	118

Malaoxon	107	91	91	110	107
Malathion	108	84	87	113	113
Methoxyfenozide	89	88	87	94	98
Metrofenone	107	100	107	121	118
Novaluron	148	147	94	132	78
Omethoate	74	65	72	71	70
Penconazole	77	70	76	79	81
Permethrin I	58	60	72	50	68
Permethrin II	62	66	76	58	62
Phenthoate	106	99	89	111	111
Phosalone	132	107	124	149	145
Phosmet	86	107	116	133	142
Phosmet Oxon	132	85	87	97	99
Pirimicarb	84	90	91	97	97
Pirimicarb Desmethyl	84	75	73	87	86
Propargite	96	97	98	106	104
Propiconazole	133	123	120	142	141
Proquinazid	89	90	91	91	86
Pyraclostrobin	92	89	84	87	96
Pyridaben	122	130	114	144	118
Pyrimethanil	56	60	64	55	63
Pyriproxyfen	90	89	91	95	96
Spinetoram	88	87	77	75	83
Spinosyn A	91	87	80	84	89
Spinosyn D	73	77	82	64	75
Spirodiclofen	123	113	103	118	105
Spiromesifen	46	58	80	55	74
Spirotetramat	143	96	116	127	132
Spirotetramat Enol	124	93	106	130	125
Spirotetramat Enol Glucoside	120	57	72	77	82
Spirotetramat Ketohydroxy	69	90	118	159	108
Spirotetramat Monohydroxy	162	123	104	148	160
Sulfoxaflor	92	67	96	103	93
Tau Fluvalinate	116	162	114	127	120
Tebuconazole	99	88	118	114	102
Tebufenozide	83	90	91	87	92
Tebufenpyrad	96	81	89	125	106
Teflubenzuron	120	162	154	93	111
Thiabendazole	74	65	78	76	70
Thiacloprid	88	77	79	89	91
Thiamethoxam	125	93	96	131	128
Thiophanate Methyl	86	99	107	112	118
Trifloxystrobin	111	104	93	97	109

When Table 4 is examined, the clean-up methods have respectively 66, 61, 92, 61, and 74 active substances in the recovery range of 70-120%. In order to get accurate results in multi-residue analyzes and to eliminate the matrix effect in chromatographic devices, matrix-matched calibrations should be used. For this reason, it has a great importance to pay attention to the fact

that the blank bee samples are not contaminated with any pesticides during the collection. In addition, it is one of the most important issues to keep the samples in dry ice for at least three hours to achieve a good homogenization. Otherwise, as the unfrozen bee bodies will remain soft, and effective homogenization process will not be possible. The main principle of the QuEChERS method is the extraction of the sample homogenized with the help of acetonitrile in foods of plant origin. However, in this method, it is necessary to add water at certain rates in samples with water content below 80% (EN 15662, 2018). According to this research, the water ratio of the body of bees is below 80% on average (Burdine and McCluney, 2019; Li *et al*., 2012). For this reason, homogenized samples were weighed and different solvent combinations were tried with different amounts of water additions. 100 active substances selected in LC-MS/MS were spiked to the blank samples at the level of 50 µg/kg. As a result of this process, the RSD values of the recovery studies with 5 replications met the condition of ≤20%. In terms of average percentage recovery values, the 1st extraction method ranks first with 72 active substances between 70-120%. Therefore, since this extraction method was found to be more successful than other methods, the clean-up was continued with this method. In addition, different clean-up methods in the QuEChERS method were tested by modifying the waiting times and adding chemicals. After the first extraction step, clean-up methods were tested for average percent recovery values. The third clean-up method is in the first place, with 92 active substances between 70-120% meeting the RSD ≤20% condition. While the number of active substances with suitable recovery values in the selected extraction method was 72, it increased to 92 with the continuation of the clean-up method. Thus, a success rate of 92% was achieved in general. The working principle of this method is formed by combining extraction and clean-up methods as follows: The samples are treated with dry ice at a temperature of -78.5°C for 3 hours to allow them to freeze thoroughly. From the homogenized samples, 10 g is weighed into 50 mL centrifuge tubes, 2.5 mL of cold water is added to them, shaken for 1 minute and waited for 5 minutes. Then, 10 mL of acetonitrile, 50 µl of ISTD (10 mg/kg Triphenyl Phosphate) are added and shaken for 2 minutes. After this stage, 4 g anhydrous magnesium sulfate, 1 g sodium chloride, 1 g trisodium citrate dihydrate and 0.5 g disodium hydrogen citrate sesquihydrate buffer salts are added to the tubes, shaken for 1 more minute and centrifuged at 3000 rpm for 5 minutes. Then, 6 mL of the supernatant part of the extract is taken into 15 mL tubes after centrifugation to purify the extract from organic acids, lipids, and sugars. Then, 900 mg magnesium sulfate, 150 mg PSA and 150 mg C18 sorbent are added and shaken for 30 seconds. Then it is centrifuged again at 3000 rpm for 5 min. 1 mL is taken from the tube and transferred to vials by passing through a 0.45 µm PTFE filter. 10 µl of 5% formic acid (prepared in acetonitrile) solution is added to the vials to be given to the LC-MS/MS device.

When this research is compared with similar research, it is seen that the results are supported. For example, Bargańska *et al*. (2018) conducted method studies to determine a broad spectrum of pesticide residues in honey bee samples after modification of QuEChERS extraction with GC-MS/MS and LC-MS/MS. The results proved that the QuEChERS protocol together with LC and GC techniques meets the requirements of green analytical chemistry and thus can be used as a tool in environmental monitoring. The recovery was found to be 85.5-103.5% for honey bee samples. Vernich *et al*. (2016) made a comparison between QuEChERS and other pesticide extraction procedures for honey and honeybee matrices. The results proved that the QuEChERS protocol is the most effective method for the detection of selected pesticides in both matrices. When reviewing at the qualitative studies in Turkey, conducted between 2006-2010 in the Laboratory of Pharmacology-Toxicology Department of Pendik Veterinary Control and Research Institute, it was aimed to evaluate the results of pesticide analysis because of suspicious bee deaths. In the suspected poisoning case, pesticide analyzes were performed on materials such as bee, honeycomb, sunflower, grass, and tree leaves. Analyzes were made qualitatively using Gas Chromatography, Gas Chromatography Mass

Spectrometer, Liquid Chromatography, and Liquid Chromatography Mass Spectrometer devices. In the analyzes, 15 insecticides, 6 naphthalene, 3 herbicides, 1 fungicide, 1 antiseptic/disinfectant and 1 growth hormone were determined (Unal *et al*., 2010). In another research between 2015-2018, 188 different dead bee samples, which came to Cukurova region for wintering, were collected from beekeepers and brought to Adana Veterinary Control Institute on suspicion of pesticide poisoning were examined. Qualitative analysis was performed with a gas chromatography (GC) device. In the examination of dead bee samples, tau-fluvalinate residues were found in 2 samples from 2015, and cypermethrin residues were found in 1 sample from 2016 (Segmenoglu, 2020).

When these researches on bees in our country are examined, it is seen that the results have been determined qualitatively. However, it has great importance to carry out quantitative analyzes in order to evaluate the results according to the maximum residue limits in pesticide analysis. Checking the suitability of pesticide analyzes in foods is carried out according to maximum residue limits. If the results are below these limits, it is considered not to be hazardous to health. It is mentioned in the literature that a similar approach is applied in pesticide analyzes to be performed on bees (Kasiotis *et al*., 2014; Naggar *et al*., 2015; *Kiljanek et al*., 2016). In these studies, LD_{50} values in bees are examined to determine if the pesticides detected cause the death of bees. In addition, six bee samples were sent to a laboratory in Germany by the Ministry of Agriculture and Forestry of Turkey for neonicotinoid analysis because the conditions of the domestic laboratories were not suitable in 2018 (Oruc and Cayci, 2019). Up to date, there is no residue laboratory in Turkey that has accreditation by the Turkish Accreditation Agency in pesticide analysis of bees.

4. CONCLUSION

It can be accepted that pesticides detected above LD_{50} values may cause bee death. Otherwise, qualitative analysis cannot be a proof that pesticides that have low toxicity cause death in bees because of non-absolute numerical results. Therefore, it would not be a correct approach to use the phrase that pesticides with low LD_{50} values and low toxicity for bees caused the death of bees.

Pesticides with LD_{50} values above the colony health of bees will create a pesticide load on bees, so the use of pesticides with low LD_{50} values and high toxicity should be avoided in plant production areas. Farmers should spray in areas with hives, especially in the evening when the bees are not flying. In addition, beekeeping activities should be carried out carefully in regions where polyculture agriculture is abundant. It is of critical importance that farmers do not apply pesticides during the flowering periods of the plants and during the hours when the bees are active. If it is not possible to do all these, colonies should be removed from these regions. Despite all the precautions to be taken, in case of bee deaths in front of the hive, samples should be taken quickly with the suspicion of possible pesticide poisoning. It is recommended to validate using this modified QuEChERS method, which is presented to the residue laboratories that will analyze the samples. It is then recommended to determine pesticide exposure in bees and to take prompt measures early without causing serious losses. This presented method will not only guide method validation studies for pesticide analysis in bees in Turkey but also avoid the need to send bee samples abroad on the grounds that laboratory conditions are not suitable.

As a result of using proposed modified QuEChERS method, it will be possible to obtain both qualitative and quantitative results in multi residue analyzes in bees. Consequently, it is possible to obtain fast, accurate and reliable results with the validation studies to be carried out with the aforementioned modified QuEChERS method, which is a combination of the 1st extraction and 3rd clean-up methods, which allows quantitative analysis of pesticides in bees. It

is recommended that the modified QuEChERS method be used in pesticide residue analyzes in bees, with its remarkable advantages such as completing the analyzes in less than six hours, working easily with the infrastructure available in all control laboratories, low analysis costs, and being easy and reliable.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Orhan Dincay: Investigation, Method development, Sample preparation, Method validation, LC–MS/MS analysis, Statistical analysis, Writing-review & editing. **Hasan Sungur Civelek:** Investigation, Writing-review & editing, Supervision.

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Chemical Composition and Antibacterial Activity of *Helichrysum italicum* (Roth) G. Don Commercial Essential Oil from Serbia

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Abstract

Medicinal plants have been preferred sources against drug-resistant pathogens recently. The antimicrobial activities of essential oils have been known for a long time. In the present research, the chemical composition and antibacterial activity of commercial essential oil of *Helichrysum italicum* (Roth) G. Don (HIE) were evaluated. Seventy components which constitute 95.7%, were identified in the oil. The major constituents of HIE were neryl acetate (15.6%), α-pinene (11.5%), and γ-curcumene (11.3%). The oil was screened for antibacterial activity by agar well diffusion and broth microdilution methods. The essential oil displayed remarkable antibacterial activity against test bacteria, except for *Escherichia coli*, which displayed resistance. The minimum inhibitory concentration (MIC) of essential oil were 5 µg/mL for *Staphylococcus aureus* and *Pseudomonas aeruginosa*, 10 µg/mL for *Bacillus cereus* and *B. subtilis*. These results suggested that the commercial oil of *H. italicum* has the potential to be used as a natural antibacterial agent.

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1. INTRODUCTION

Bacteria have been accepted as the main reasons for diverse human infections. Although effective antimicrobials have been improved, microorganisms have developed resistance against the presently launched antibiotics. This situation has required the improvement of novel antimicrobial drugs. Medicinal plants and herbs have been preferred sources against drug-resistant pathogens recently. The antimicrobial activities of essential oils have been known for a long time (Chopra, 2007). *Helichrysum italicum* (Roth) G. Don is an aromatic Mediterranean plant and belongs to the family Asteraceae. *H. italicum* (immortelle in Italian) essential oil is used mainly in cosmetic products particularly skincare products that stimulate blood circulation in the skin, enable regeneration, and have anti-ageing effects. The dried flowers of the plant are used as choleretic, diuretic, and expectorant in traditional medicine (Chinou *et al*., 1996). The plant has antimicrobial, anti-inflammatory, antioxidant, anti-viral, and anti-HIV properties (Nostro *et al*., 2001; Sala *et al*., 2002; Nostro *et al*., 2003; Appendino *et al*., 2007; Rosa *et al*., 2007). The HIE has also been widely studied. The essential oils displayed variation in their major compounds due to distinct geographical regions, climatic and environmental conditions, vegetative stages, plant parts, and extraction methods (Aćimović *et al*., 2021). The main compounds of the oil were α-pinene, β-selinene, γ-curcumene, neryl acetate, citronellol isobutanoate, α-*trans*-bergamotene, β-acoradiene, rosifoliol, nerol, neryl propionate, linalool, α-terpineol, α-cedrene, α-curcumene, spathulenol, β-caryophyllene, geranyl acetate, limonene, β-eudesmene, β-bisabolene, 4,6,9-trimethyldec-8-en-3,5-dione, *cis*-α-guaiene, nerolidol-E,

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eudesmen-7-(11)-en-4-ol, 2-methyl-cyclohexyl pentanoate, and *iso*-italicene epoxide (Blažeković *et al*., 1995; Bianchini *et al*., 2001; Angioni *et al*., 2003; Paolini *et al*., 2006; Stupar *et al*., 2014; Ćavar Zeljković *et al*., 2015; Melito *et al*., 2016; Djihane *et al*., 2017; Staver *et al*., 2018; Tzanova *et al*., 2018; Andreani *et al*., 2019; Fraternale *et al*., 2019; Gismondi *et al*., 2020; Oliva *et al*., 2020; Aćimović *et al*., 2021). The study aimed to determine the chemical composition and antibacterial activity of HIE.

2. MATERIAL and METHODS

2.1. Source of Material

Commercial essential oil was purchased from local market in Serbia.

2.2. GC-FID and GC-MS analyses

The GC-MS analysis was employed with an Agilent 5975C Inert XL EI/CI MSD system in EI mode. The essential oil was diluted 1:10 (v:v) with *n*-hexane. MS transfer apparatus and injector temperatures were set at 250˚C. In GC-MS analyses, the capillary column type was Innowax FSC (60 m × 0.25 mm, 0.25 µm film thickness) and the carrier gas was helium with a flow rate of 1 mL/min. The injection was done in split mode. The oven temperature was arranged to 60°C for 10 minutes and increased to 220°C at 4°C/min., where the temperature was kept stable for 10 minutes and the temperature was increased to 240°C at 1°C/min. The mass spectra were saved at 70 eV with the mass range *m*/*z* 35 to 425. The relative percentages of the separated compounds were calculated from the integration of the peaks in MS chromatograms. The constituents of essential oils were determined by comparison with GC-MS libraries (Wiley 8th Ed. and NIST14), and their relative retention indices (RRI) were obtained by *n*-alkanes series (C5 to C30) to the literature. The GC analyses were done with an Agilent 6890N GC system. FID detector temperature was set to 300°C and the same operational conditions were applied to a duplicate of the same column used in the GC-MS analyses. Simultaneous autoinjection was used to obtain the same retention times. The relative percentage amounts of the separated compounds were calculated from integration of the peaks in the FID chromatograms (Servi *et al*., 2021).

2.3 Antibacterial Activity

The antibacterial activity of the essential oils was evaluated by using disc diffusion and broth microdilution assays according to a modified by Azaz *et al*. (2005) *E. coli* ATCC 14169, *S. aureus* ATCC 25923, *B. subtilis* ATCC 19659, *B. cereus* ATCC 14579, and *P. aeruginosa* ATCC 27853 were used as test microorganism. The stock solution of the essential oils was prepared with 10% dimethyl sulfoxide (DMSO). Serial dilution of essential oil was done on 96-well microplates with using Mueller Hinton Broth (MHB). Bacteria were standardized in MHB according to McFarland No:0.5. The microorganism suspension was diluted to a final concentration of 5 x 10^5 CFU/mL. Bacterial cultures were mixed with different concentrations of essential oils on microplates and were incubated 24 h at 37ºC. The measurement of minimum inhibitory concentration (MIC) of essential oils was assessed visually by the change in the colour of the resazurin indicator. The colour changes from pink or colourless indicated the growth of bacteria.

3. RESULTS and DISCUSSION

Seventy components which constitute 95.7% were identified in the oil. The major constituents were neryl acetate (15.6%), α-pinene (11.5%), and γ-curcumene (11.3%) (Figure 1 and Table 1). The dominant groups of oil were sesquiterpenes (43.0%), oxygenated monoterpene (21.8%), and monoterpenes (16.7%). The previous studies determined that *H. italicum* essential oil had sesquiterpene, monoterpene and oxygenated monoterpene as

dominant groups. In the current study, *H. italicum* oil displayed similar major groups to the past studies (Staver *et al*., 2018; Aćimović *et al*., 2021; Mollova *et al*., 2020). Aćimović *et al*. (2021) reported that γ-curcumene (13.6%), β-selinene (12.2%), and α-pinene (11.8%) were determined as the main components in the essential oil of *H. italicum* cultivated in Serbia. Nerly acetate was found threefold lower, and β-selinene was determined almost threefold higher in the current study. Staver *et al*. (2018) indicated that γ-curcumene and α-pinene (21.6%) were the dominant components of *H. italicum* essential oil from Croatia. There are quantitative differences in the main compounds between previous and current studies. In a study conducted by Djihane *et al*. (2017), α-cedrene (13.6%), α-curcumene (11.4%), and geranyl acetate (10.1%) were determined as dominant constituents of *H. italicum* essential oil from Algeria. The major constituents of the previous study were not determined in the oil of the current study. Oliva *et al*. (2020) reported that β-eudesmene (21.7%), β-bisabolene (19.9%), α-pinene (16.9%), and neryl acetate (10.7%) were dominants components in the oil from Montenegro. β-eudesmene and β-bisabolene were not found in the oil of the current study. Mastelic *et al*. (2005) reported that the essential oil of *H. italicum* from Croatia was fractionated in terpene and terpenoid fractions. The major compounds of essential oil were neryl acetate (11.5%) and α-pinene (10.2%). The terpene fraction had α-pinene (20.6%) and α-cedrene (20.5%) as the main compounds. Neryl acetate (23.2%) and 2-methylcyclohexyl pentanoate (12.9%) were the main compounds of the terpenoid fraction. α-cedrene and 2-methylcyclohexyl pentanoate were not determined in the oil of the current study. Also, γ-curcumene was not found in the oil of the previous study. γ-curcumene (14.1%), β-selinene (11.3%), and α-curcumene (10.4%) were the dominant components of *H. italicum* essential oil from Montenegro (Šćepanović *et al*., 2019). α-curcumene was not determined and β-selinene was found threefold lower in the present study. Also, α-pinene (0.7%) was found in a meagre amount in the oil of the previous study. Mollova *et al*. (2020) reported that the main component of plant origin from France was neryl acetate (33.9%). α-pinene (20.8%) and γ-curcumene (16.5%) were the dominant compounds of the plant origin from Bosnia. There are quantitative differences in the main compounds from current and previous studies. The quantitative composition of the volatile oils of many aromatic plants is remarkably affected by the harvesting time, plant age, vegetative stages, and product density (Chauhan *et al*., 2010; Melito *et al*., 2015; Tzanova *et al*., 2018; Aćimović *et al*., 2021).

Table 1. Essential oil composition of *Helichrysum italicum*.

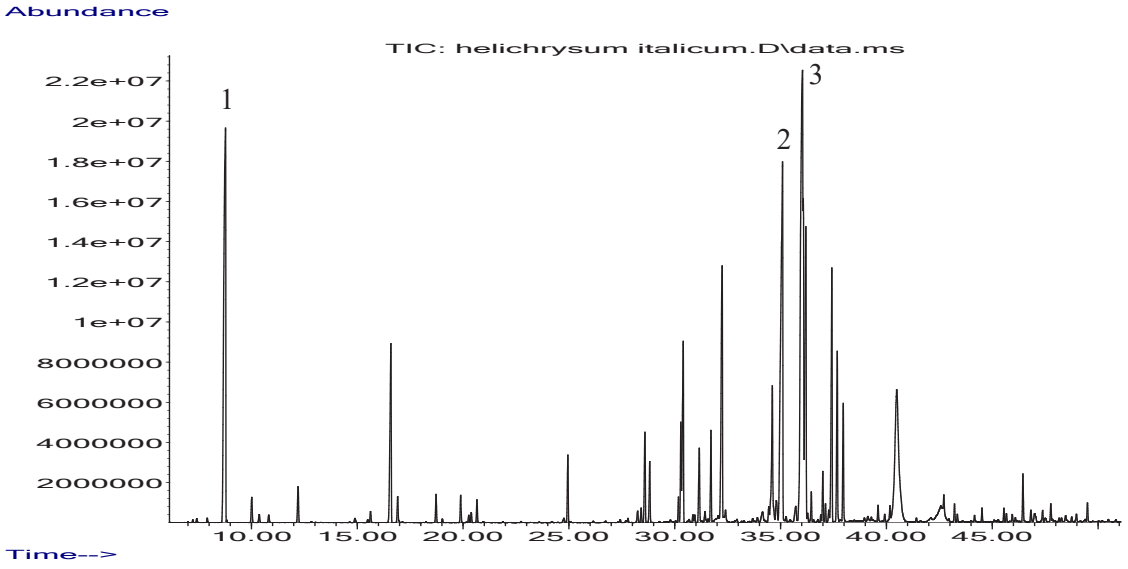
RT[1]	RRI[2]	RRI Lit.[3]	Compound	%
8.763	1023	1032	α-Pinene	11.5
10.018	1055	1072	α-Fenchone	0.4
10.371	1064	1076	Camphene	0.1
10.822	1075	1087	2-Hexanone	0.1
12.206	1109	1118	β-Pinene	0.6
15.631	1180	1188	α-Terpinene	0.2
16.587	1200	1203	Limonene	3.4
16.913	1207	1213	1,8-Cineol	0.4
18.727	1247	1255	γ-Terpinene	0.4
19.89	1273	1280	*p*-Cymene	0.4
20.273	1282	1286	2-Methylbutyl-2-methylbutyrate	0.1
20.389	1284	1290	α-Terpinolene	0.1

20.66	1290	1293	Isobutyl angelate	0.3
24.769	1391	1398	2-Nonanone	0.1
24.953	1396	1395	2-Methyl butyl angelate	0.9
27.796	1474	1480	Nerol oxide	0.1
28.251	1487	1493	α-Ylangene	0.2
28.418	1491	1492	Cyclosativene	0.2
28.594	1496	1497	α-Copaene	1.4
30.295	1548	1553	Linalool	1.3
30.399	1551	1553	Italicene	3.1
30.868	1565	1587	Di-*epi*-α-cedrene	0.1
30.953	1568	1571	Isoitalicene	0.1
31.163	1574	1568	*Trans*-α-bergamotene	1.2
31.435	1583	1589	Isocaryophyllene	0.2
31.716	1591	1594	*Trans*-β-bergamotene	1.2
32.057	1602	1604	2-Undecanone	0.3
32.236	1608	1612	β-Caryophyllene	5.3
32.405	1613	1617	Guaia-6,9-diene	0.3
33.913	1663	1667	Amorpha-4,11-diene	0.1
34.156	1671	1668	(*Z*)-β-Farnesene	0.4
34.443	1680	1687	α-Humulene	0.2
34.614	1686	1690	γ-Selinene	2.8
34.689	1688	1690	α-Acoradiene	0.1
35.089	1702	1704	γ-Curcumene	11.3
35.267	1708	1719	Borneol	0.1
35.721	1724	1718	*Cis*-β-guaiene	0.4
36.029	1735	1733	Neryl acetate	15.6
36.079	1736	1742	β-Selinene	3.3
36.202	1741	1744	α-Selinene	5.8
36.909	1765	1771	(*E*)-γ-Bisabolene	0.1
37.003	1769	1772	δ-Cadinene	0.7
37.128	1773	1776	γ-Cadinene	0.2
37.286	1779	1785	7-*Epi*-α-selinene	0.2
37.425	1784	1786	ar-Curcumene	4.0
37.681	1792	1772	Neryl isobutyrate	2.4
37.966	1803	1808	*Cis*-geraniol	1.6
39.132	1846	1853	*Cis*-calamenene	0.1
39.614	1864	1857	Neryl 2-methylbutanoate	0.2
39.929	1875	1892	Italicene ether	0.1
40.174	1885	1871	Neryl isovalerate	0.3
40.498	1897	1869	2,4,6,9-Tetramethyldec-8-ene-3,5-dione	8.4

42.721	1983	1950	3,5,7,10-Tetramethylundec-9-ene-4,6-dione	0.6
43.227	2003	2008	Caryophyllene oxide	0.2
43.361	2008	2000	Citronellyl hexanoate	0.1
44.173	2042	2050	(*E*)-Nerolidol	0.1
45.561	2098	2103	Guaiol	0.2
45.955	2115	2214	Phenyl ethyl tiglate	0.1
46.46	2137	2144	Rosifoliol	0.7
46.837	2153	2161	Neointermedeol	0.2
47.016	2160	2170	β-Bisabolol	0.2
47.389	2176	2185	γ-Eudesmol	0.2
47.778	2193	2187	τ-Cadinol	0.2
47.907	2199	2209	Tau-muurolol	tr
47.985	2202	2219	δ-Cadinol	tr
48.479	2224	2248	Bulnesol	0.1
48.766	2237	2250	α-Eudesmol	0.1
48.988	2247	2257	β-Eudesmol	0.1
49.508	2270	2273	Selin-1-en-4α-ol	0.3
			Monoterpenes	16.7
			Monoterpenoids	21.8
			Sesquiterpene	43.0
			Sesquiterpenoids	3.2
			Others	11.0
			Total identified	95.7

[1]R.T: Retention time; [2]RRI: Relative retention time; [3]RRI Lit.: Relative retention time in the literature.

Figure 1. GC-MS chromatogram of HIE (**1:**α-Pinene; **2:**γ-Curcumene; **3:**Neryl acetate).



The commercial oil was screened for antibacterial activity against five test bacteria by disc diffusion and broth microdilution methods (Table 2 and Table 3). Vieira *et al*. (2017)

reported that the MIC values lower than 100 µg/mL, between 100 and 500 µg/mL, between 500 and 1000 µg/mL, and higher than 1000 µg/mL correspond to an essential oil that is a promising, moderately active, weak, and inactive antimicrobial, respectively. When our results compared with the previous study, the essential oil was inactive against *E. coli*. However, the essential oil displayed promising activity against *S. aureus*, *P. aeruginosa* (5 µg/mL), *B. cereus* and *B. subtilis* (10 µg/mL).

Table 2. The inhibition zone diameters (mm) of essential oil.

	Gram-positive bacteria			Gram-negative bacteria	
	Staphylococcus aureus	*Bacillus subtilis*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia coli*
Essential oil	10	12	13	10	-

Table 3. The MIC values (µg/mL) of essential oil.

	Gram-positive bacteria			Gram-negative bacteria	
	Staphylococcus aureus	*Bacillus subtilis*	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Escherichia coli*
Essential oil	5	10	10	5	-

There are reports in the literature on the antibacterial activity of essential oil of *H. italicum*. Aćimović *et al*. (2021) reported that the essential oil of *H. italicum* cultivated in Serbia was tested against 16 strain isolates by broth dilution method. The oil displayed low or no activity against test bacteria. Staver *et al*. (2018) indicated that *H. italicum* essential oil from Croatia was evaluated against Gram (+), Gram (-) bacteria and one yeast. The oil displayed weak and moderate activity against test bacteria. In a study conducted by Djihane *et al*. (2017), the MIC values of essential oil were between 0.79-50.6 µg/mL. Oliva *et al*. (2020) reported the volatile oil showed no antimicrobial activity against test microorganisms. Mastelic *et al*. (2005) reported that the essential oil, terpene, and terpenoid fractions were evaluated for antimicrobial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, and *Candida albicans*. The essential oil and terpenoid fraction were more effective against *S. aureus* (5.0 µL/mL) and *C. albicans* (5.0 µL/mL) than the terpene fraction. The antimicrobial activity of *H. italicum* essential oil from Montenegro was studied against nine test microorganisms. The oil displayed antimicrobial activity against *B. subtilis* with the 1.4 µg/mL MIC value (Šćepanović *et al*., 2019). Mollova *et al*. (2020) reported that the essential oils of *H. italicum* plant origin from Bosnia and France were studied for antimicrobial activity by the disc diffusion method. The oil of the plant origin from France was more effective against *S. aureus* and *B. subtilis* than the oil of the plant origin from Bosnia. The MIC values and inhibition zone diameters of the essential oils of the previous studies had a different profile from the current study due to the different percentages of the main compounds (Chauhan *et al*., 2010; Melito *et al*., 2016; Tzanova *et al*., 2018; Aćimović *et al*., 2021).

4. CONCLUSION

The chemical composition of *H. italicum* commercial oil was determined, and GC-MS results indicated qualitative similarity and quantitative diversity in the essential oil components. Antimicrobials from natural sources have gained extensive significance recently in fighting the worldwide challenge of antibiotic resistance. Essential oils showed antimicrobial activity

against tested bacteria species. The results of the antibacterial activity suggested that the essential oil of *H. italicum* has the potential to be used as a natural antibacterial agent.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Esra Yildirim Servi: Writing, laboratory work, editing, validation. **Huseyin Servi:** Laboratory work, writing.

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Genome Wide Analysis and Characterization of *NPR-like* Gene Family of *Phaseolus vulgaris* L.

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Abstract

Nonexpressor of pathogenesis-related genes 1 (*NPR1*) could be found in the regulation of pathways of salicylic acid (SA) signal transduction and systemic acquired resistance (SAR) in plants. It has been found that the members of *NPR-like* gene family are interconnecting with biotic and abiotic stresses in several plants, but it has not yet been performed in common bean. In this study, *in silico* analyzes of *Phaseolus vulgaris* L. *NPR-like* gene family members were performed at the genome level. As a result of these *in silico* analyzes, four *NPR1* genes were identified and it was found that these genes have been located on four different *P. vulgaris* chromosomes. The length of *PvNPR-like* proteins ranges from 475-590 amino acid residues. The theoretical isoelectric point (pI) range were found between 5.75-6.36 and all show acidic properties. As a result of phylogenetic analyzes performed between *NPR-like* genes of *P. vulgaris*, *Glycine max* (L.) Merr and *Arabidopsis thaliana* (L.) Heynh. plants, *NPR-like* genes were found to be divided into three different groups (A, B and C). It has been determined that *PvNPR-like* genes contain *cis-acting* elements that function in many biological processes, including stress and hormone responses. Differences in gene expression levels under salt and drought stress indicated that *PvNPR-like* genes respond differently under stress conditions. The outcomes of this study will supply the needed basics for understanding the function and evolutionary history of *PvNPR-like* genes.

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1. INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is an annual legume with a genome size of 437 Mb (Schmutz *et al*., 2014). Contenting of *P. vulgaris* is composed of high lysine rich, carbohydrates, fiber and minerals (Siddiq and Uebersax, 2022). Common bean production is restricted due to diverse environmental factors. *Sclerotinia sclerotiorum, Aspergillus* and *Cladosporium* fungi are also pathogens that seriously affect bean production and yield (Carvalho *et al*., 2011). As with biotic stresses, abiotic stresses such as high salt, drought and low temperature strongly affect plant productivity (Bajaj *et al*., 1999). In order to survive under these stress conditions, plants show many physiological, biochemical and molecular changes and form various responses to stress. When the stress mechanism in plants was examined at the molecular level, it was concluded that there were changes in the expression of early and delayed response genes in response to stress (Munns and Tester, 2008). One of these responses, systemic

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acquired resistance (SAR), is a long-term plant defense mechanism that provides broad resistance against viral, bacterial and fungal pathogens and increases the expression level of pathogenesis-related genes (PRs) (Ryals *et al*., 1996). *NPR1* gene is needed for PR gene expression and transduction of SA (salicylic acid) signal that activates SAR (Dong, 2001). *NPR1* is the master regulator of SAR in plants (Fu and Dong, 2013). *NPR1* has an N-terminal *BTB/POZ* domain, an ankyrin repeat, and a C-terminal nuclear localization signal (Aravind and Koonin, 1999; Kinkema *et al*., 2000). The first studies on *NPR* genes were carried out in the 1990s. *NPR1*, one of the two classes identified in the *Arabidopsis* mutant, was found to be induced by SA and altered the expression of PR genes (Cao *et al*., 1994; Cao *et al*., 1998). It was then thought that *NPR1* interacts with members of the TGA transcription family and in this way affects PR gene expression (Zhou *et al*., 2000). In recent years, *NPR1* was found to bind SA and interact with subsets of the TGA gene family to perform its transcriptional co-activator function (Wu *et al*., 2012; Ding *et al*., 2018). Transcription factors (TFs) are proteins binding to DNA in a very specific state and suppress or improve gene expression. In response to a wide variety of stimuli, TFs are involved in many important biological functions, from cell cycle and physiological responses to cell differentiation and development (Kızılkaya *et al*., 2020). It is known that approximately 30 families of transcription factors have been identified and classified according to conserved motifs encoding DNA binding sites (Inal *et al*., 2017; Ilhan, 2018). TGA factors are basic leucine-zipper (bZIP) transcription factors that are classified according to their ability to bind to the activating sequence-1 (as-1) region of the CaMV 35S promoter (Katagiri *et al*., 1989). Both as-1 and associated elements contain the TGACC core sequence and often respond to stress or phytohormones, particularly SA, jasmonic acid (JA), and auxin (Xiang *et al*., 1996). Before the initiation of SAR, *NPR1* is present in the cytoplasm in a homooligomeric complex. SA accumulation by stimulation of SAR triggers a redox change that leads to monomerization of *NPR1* as a result of breaking the disulfide bonds between molecules. *NPR1* in monomeric form is transported to the nucleus and activates PR gene expression (Cao *et al*., 1997). Similarly, *NPR1* interacts with the HSFA1 transcription factor to activate the expression of genes regulated by HSFA1, a key regulator of the heat shock response to cold (Olate *et al*., 2018). Six *NPR-like* genes have been identified in the *A. thaliana* genome (Hepworth *et al*., 2005). *NPR-like* genes have been identified in *Oryza sativa* L. (Sugano *et al*., 2010), *Malus domestica* Borkh. (Zhang *et al*., 2016), *Fragaria spp.* (Shu *et al*., 2018), *Triticum aestivum* L., *T. urartu* Thumanjan ex Gandilyan, *T. dicoccoides* (Koern. ex Asch. & Graebn.) Schweinf. (Liu *et al*., 2019), *Gossypium* spp. (Agarwal *et al*., 2020), *Brassica juncea* (L.) Czern. (Wang *et al*., 2021), *Vitis vinifera* L. (Goyal *et al*., 2021) and *Pyrus bretschneideri* Rehder (Wei *et al*., 2021). As a result of this study, no information was found about the genome-wide analysis of the *NPR-like* gene family in common bean plant. The perspective of this study was to perform genome-wide characterization of *NPR-like* gene family members in *P. vulgaris* genome by *in silico* methods and to determine ortholog-paralog relationships between *P. vulgaris* L. (NCBI taxo. ID: 2885), *G. max* (L.) Merr., 1917 (NCBI taxo. ID: 3847) and *A. thaliana* (L.) Heynh., 1842 (NCBI taxo. ID: 3702) *NPR-like* genes. In addition, it was aimed to determine the gene expression levels of *NPR-like* proteins under salt and drought stress.

2. MATERIAL and METHODS

2.1. Identification of *NPR-like* Gene Family Proteins in the Common Bean (*P. vulgaris*) Genome

The protein sequences of the *NPR-like* gene family in common bean genome were obtained from the Phytozome database v13 via the accession number (PF12313) was found in the Pfam database (https://phytozome-next.jgi.doe.gov/). To identify all *NPR-like* proteins likely to be present in common bean genome, blastp in the Phytozome database v13 and Hidden Markov Model (HMM) were scanned in the *P. vulgaris* (Schmutz *et al*., 2014), *A. thaliana*

(Lamesch *et al*., 2012) and *G. max* (Valliyodan *et al*., 2019) genome with available parameters. Presence of *BTB/POZ*, ankyrin repeat and *NPR-like*_C domain in related sequences were searched by using the HMMER database (http://hmmer.org/). The amino acid numbers, molecular weight, theoretical isoelectric point (pI), instability index and stability of the ingested *NPR-like* proteins were determined with the help of the "ProtParam tool" (https://web.expasy.org/protparam/). Subcellular localizations of *NPR-like* genes were predicted by the WoLF PSORT database (Horton *et al*., 2007).

2.2. Identification of Physical Locations, Gene Duplication, Structure and Conserved Motifs of *NPR-like* Genes

Gene Structure Display Server v2.0 was used to obtain information about the intron-exon regions of *PvNPR-like* proteins (Hu *et al*., 2015). DNA (CDS) sequences encoded together with genome sequences were used to find the region of *PvNPR-like* genes. The sizes and chromosomal locations of *PvNPR-like* genes were determined using the Phytozome database v13. *PvNPR-like* genes were scanned in all bean chromosomes and the chromosomes were detected and drawn with the help of MapChart program (Voorrips, 2002). Non-synonymous ratios (Ka), synonymous ratios (Ks), and evolutionary strains (Ka/Ks) among *PvNPR-like* gene duplications were calculated by using the PAML tool (Yang, 2007) in the PAL2NAL interface (http://www.bork.embl.de/pal2nal/) (Suyama *et al*., 2006). The "Multiple EM for Motif Elimination (MEME) Tool" was used to detect additional conserved motifs of *PvNPR-like* proteins (Bailey *et al*., 2006). Limits for minimum/maximum width were set 6 to 50 respectively and maximum number of motifs to be found was set 10. The motif zones range from 2 to 300. Any number of repetitions was chosen as the region distribution. The motifs determined as a result of the analysis were scanned using the available settings in the InterProScan database (Quevillon *et al*., 2005).

2.3. Sequence Alignment and Phylogenetic Analyzes

Phylogenetic analyzes were performed by using the Neighbor-joining (NJ) method with 1000 replicated bootstrap values. Protein sequences of *PvNPR-like* aligned by ClustalW in the MEGA software (Thompson *et al*., 1997). Phylogenetic tree was obtained by MEGA 11 program using *NPR-like* proteins from *P. vulgaris, G. max* and *A. thaliana* (Tamura *et al*., 2011). The phylogenetic tree is shaped with the "Tree of Interactive Life (iTOL)" interface (Letunic and Bork, 2011).

2.4. Synteny Analysis

The sequences of orthologous proteins of plants which are *P. vulgaris*, *G. max* and *A. thaliana* have been obtained from the Phytozome database v13 and then the synteny map was created using TBtools software (Chen *et al*., 2020).

2.5. Promoter analysis of *PvNPR-like* genes

To scan *cis-acting* elements, the 5′ upstream sequences (2kb DNA sequence) retrived from Phytozome database v13 was analyzed with the PlantCARE database (Lescot *et al*., 2002) and shaped with the TBtools program.

2.6. Homology Modeling of *PvNPR-like* Proteins

3D structures of *PvNPR-like* protein sequences were obtained through the Phyre2 (Kelley *et al*., 2015) database. The best 3-dimensional match was selected by looking at the reliability rates of the models obtained as a result of the analysis.

2.7. *PvNPR-like* Gene Ontology Analysis and Protein-Protein Interactions

The STRING database (https://string-db.org/) was used to detect functional and physical interactions of *PvNPR-like* protein-protein interactions (PPIs). PPIs were shaped using Cytoscape program (Shannon *et al*., 2003). Ontology data of *PvNPR-like* genes were used to obtain functional information of *PvNPR-like* proteins using the Omics Box program (https://www.biobam.com/omicsbox).

2.8. In-silico Gene Expression Analysis

Illumina RNA-seq datasets were obtained with (https://www.ncbi.nlm.nih.gov/sra) database. Expression profiles of *PvNPR-like* genes were analyzed from SRA specific tissue libraries obtained from the plant under drought (drought control: SRR8284481, drought treatment: SRR8284480) (Gregorio *et al*., 2020) and salt (salt control: SRR957668, salt treatment: SRR958469) stress (Hiz *et al*., 2014) (https://www.ncbi.nlm.nih.gov/sra). In silico expression profiles were calculated in RKPM units with Cufflinks (Trapnell *et al*., 2012). RKPM values were calculated on the basis of log2, and a heatmap was created with the software Orange (http://orange.biolab.si).

3. RESULTS and DISCUSSION

3.1. *NPR-like* Gene Family Members Identification in the Genome of *P. vulgaris*

Keyword search was performed on the common bean genome in Phytozome database v13 with Pfam accession number (Pfam ID) obtained from Pfam database. As a result of this analysis, four genes with *NPR-like* protein homology were found (Supplementary File 1). The presence of *BTB/POZ* (broad complex, tramtrack, and bric-a-brac/ poxvirus and zinc finger), ankyrin repeat, and the *NPR-like*_C domain was confirmed by the HMMER database. *BTB/POZ* and ankyrin repeat were found in all genes, while the *NPR-like*_C domain was found only in *PvNPR2* and *PvNPR4*. Four *NPR-like* genes identified from the common bean (*P. vulgaris*) genome were listed in Table 1. In addition, the molecular weights, amino acid numbers, theoretical isoelectric points, instability index, stability status and intracellular localization of these genes were given in Table 1. *PvNPR-like* genes were located on chromosomes 1, 6, 7 and 8 of the common bean (Figure 1).

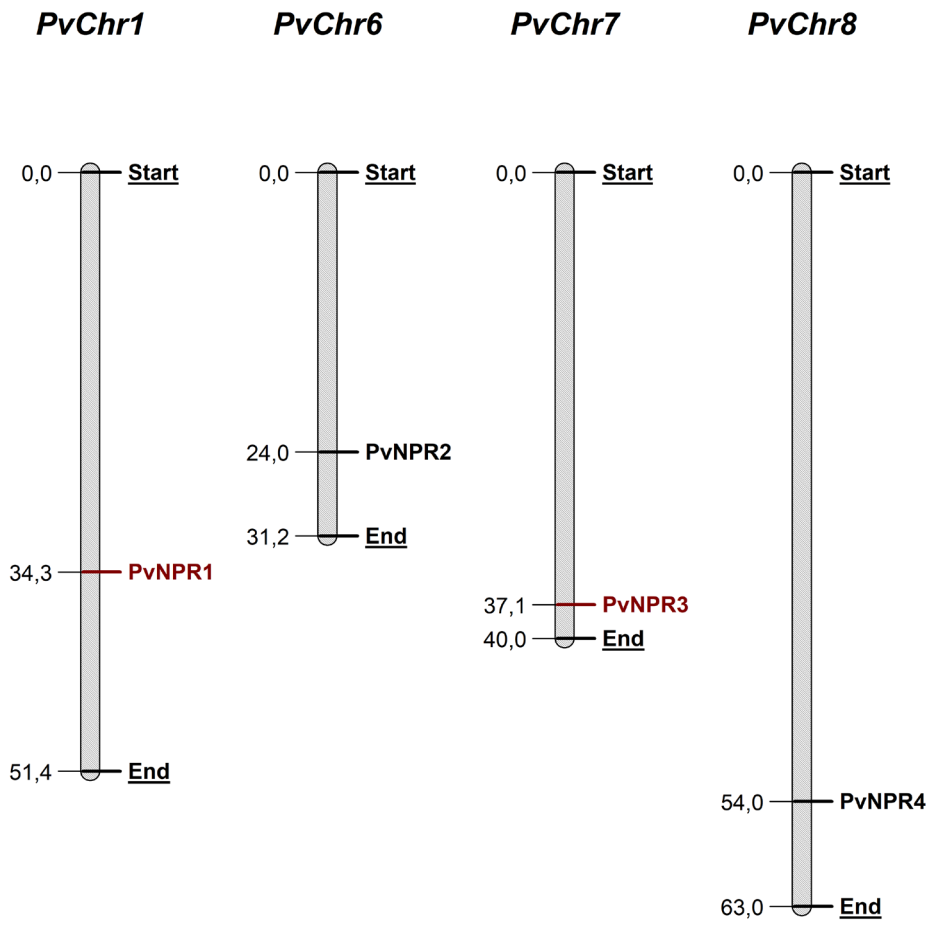
In previous studies, *NPR-like* genes were found in *Gossypium hirsutum* L. (5), *Gossypium barbadense* L. (6) (Agarwal *et al*., 2020), *T. aestivum* (17), *T. urartu* (5), *T. dicoccoides* (12), *Ae. tauschii* (6) (Liu *et al*., 2019) and *Brassica juncea* var. *tumida* M. Tsen & S.H. Lee (19) (Wang *et al*., 2021). *BTB/POZ* and *NPR-like*_C domains and ankyrin repeats were also detected in the genes.

The amino acid length of *PvNPR-like* proteins ranged from 475-590. Among them, *PvNPR2* had the longest amino acid chain (590) and it was found that the gene encoding the shortest amino acid chain (475) was *PvNPR1*. The theoretical isoelectric point (pI) was found be between 5.75-6.36 and all of them were shown acidic properties. The highest pI value (6.36) was in *PvNPR2* while the lowest pI value (5.75) was in *PvNPR4*. When the molecular weights were examined, *PvNPR1* (52.14 kDa) had the lowest molecular weight and *PvNPR4* (65.93 kDa) had the highest molecular weight. *PvNPR-like* proteins have been found to be unstable. According to WoLFPSORT database, it was determined that the *PvNPR-like* genes were predominantly found in the nucleus but were also found in other subcellular components. In previous studies, it was determined that the amino acid length of *B. Juncea NPR1* genes ranged from 569-616, the molecular weights were between 63.41-68.84 kDa, and theoretical isoelectric point was between 5.71-5.89 (Wang *et al*., 2021). The amino acid length of *P. bretschneideri NPR1* genes was found to be 498-591, the molecular weight 54.51-66.25 kDa, and theoretical isoelectric point varied from 5.7-6.4 (Wei *et al*., 2021).

Table 1. Information on *PvNPR-like* proteins found in the common bean genome.

Gene Name	Phytozome ID	Chr No	aa Length	MW (kDa)	pI	Instability index	Stability	Subcellular Localization (WolfPsort)
PvNPR1	Phvul.001G124100	1	475	52.14	6.10	48.13	unstable	nucl: 5, cyto: 4, chlo: 3, plas: 1, E.R._vacu: 1
PvNPR2	Phvul.006G131400	6	590	65.90	6.36	44.85	unstable	nucl: 7, cyto: 5, chlo: 2
PvNPR3	Phvul.007G248000	7	479	52.25	5.92	44.45	unstable	chlo: 4, nucl: 2, plas: 2, vacu: 2, E.R.: 2, cyto: 1, mito: 1
PvNPR4	Phvul.008G195900	8	588	65.93	5.75	49.46	unstable	nucl: 11, mito: 1, plas: 1, cysk: 1

Figure 1. Distribution of *PvNPR-like* genes in bean chromosomes (Red indicates segmental duplicated genes).



Gene duplication events are very important for the development and further expansion of gene families (Mehan *et al*., 2004). For this aim, analysis was performed to detect tandem and segmental duplication events between *PvNPR-like* genes. While no tandem duplication was detected, and segmentally duplicated gene pairs were found between *PvNPR1* and *PvNPR3* genes. In a previous study, a total of 17 segmentally duplicated genes were identified in *NPR-like* genes. In that study, six genes in the A genome, five genes in the B genome, and six genes in the D genome of *T. Aestivum* (Liu *et al*., 2019).

Whole genome folding or polyploidy is a common phenomenon in nature. The duplicated genes due to polyploidy provide important raw genetic material for adaptive evolution

(Kasapoglu *et al*., 2020). The Ka/Ks ratio of segmentally duplicated gene pairs (*PvNPR1-PvNPR3*) was calculated as 0.0478 (Table 2). This result exhibited that purifying selection occurred during the evolution of the gene sequence since the Ka/Ks ratio was less than 1 (Juretic *et al*., 2005). The *PvNPR1* and *PvNPR3* genes diverged approximately 120.65 million years ago.

Table 2. *PvNPR-like* gene duplications.

Gene 1	Phytozome Id	Gene 2	Phytozome Id	Ka	Ks	Ka/Ks	Mya (Million Years Ago)
PvNPR1	Phvul.001G124100	PvNPR3	Phvul.007G248000	0.076	1.583	0.048	120.65

3.2. Gene structure, conserved motifs and phylogenetic analyzes of *PvNPR-likes*

To determine the relationship between *NPR-like* proteins, a phylogenetic tree was constructed by using the sequence information of the *NPR-like* genes of *P. vulgaris, G. max* and *A. thaliana*. The phylogenetic tree was plotted using the neighbor-joining method with a 1000 repetitive bootstrap value according to the amino acid sequence of the *NPR-like* proteins. *NPR-like* proteins were divided into 3 groups, named A, B, and C. *PvNPR1* protein showed orthology with the *GmNPR7* (Glyma.03G128600) protein. *PvNPR2* protein showed orthology with the *GmNPR2* (Glyma.09G020800) and *GmNPR5* (Glyma.15G127200) proteins. *PvNPR3* protein showed orthology with the *GmNPR6* (Glyma.02G188700) protein. *PvNPR4* showed orthology with the *GmNPR1* (Glyma.02G283300) and *GmNPR4* (Glyma.14G031300) proteins (Figure 2).

The *NPR-like* proteins of *G. hirsutum, G. barbadense, Gossypium raimondii* Ulbr., *Gossypium arboreum* L., *A. thaliana, Persea americana* Mill., *Carica papaya* L., *G. max, M. domestica, Populus trichocarpa* Torr. & A.Gray ex. Hook., *Lycopersicon esculentum* Mill., *Sorghum bicolor* (L.) Moench, *O. sativa, Zea mays* L., *Physcomitrella patens* (Hedw.) Bruch & Schimp. And *Selaginella moellenfii* Hieron.were divided into three different classes (Agarwal *et al*., 2020). Similarly, as a result of phylogenetic analyzes performed with *P. bretschneideri* in 20 different monocotyledons and dicotyledonous species, *NPR-like* proteins were found to be divided into 3 different classes (Wei *et al*., 2021).

With the MEME Suite (v5.3.3) (Bailey *et al*., 2006) program, 10 conserved motifs were found in *PvNPR-like* proteins. The amino acid lengths of the identified motifs varied from 21-50. A total of 9 motifs were detected in all *PvNPR-like* genes and the sequence information of these motifs was given in Table 3 and Figure 3. Out 6 of 9 motifs (1, 2, 3, 4, 5 and 6) were found in all genes. Motif 7 and 8 were only found in *PvNPR2* and 4, while motif 9 and 10 were found in *PvNPR1* and 3. In addition, as a result of InterProScan analysis with the identified motifs, it was found that there was a Regulatory protein NPR5/6 domain in motifs 1, 3 and 4 and a Regulatory protein NPR domain in motifs 7 and 8. In the conserved motif analysis performed in *P. bretschneideri NPR* genes, it was observed that these 6 motifs are located in all genes. These motifs can be used to detect the presence of the *NPR-like* gene family (Wei *et al*., 2021).

Exon and intron numbers were determined as a result of analysis of *NPR-like* genes with Gene Structure Display Server v2.0. As a result of the analysis, at most 3 (*PvNPR2* and *PvNPR4*) and at least 1 (*PvNPR1* and *PvNPR3*) intron region were detected, while at most 4 (*PvNPR2* and *PvNPR4*), and at least 2 (*PvNPR1* and *PvNPR3*) exon regions were detected. *PvNPR1* and *PvNPR3* genes show homology with *AtNPR5* and *AtNPR6* genes, which have the same exon-intron structure (Figure 4).

Figure 2. Phylogenetic tree plotted with *NPR-like* proteins of *A. thaliana, G. max* and *P. vulgaris.*

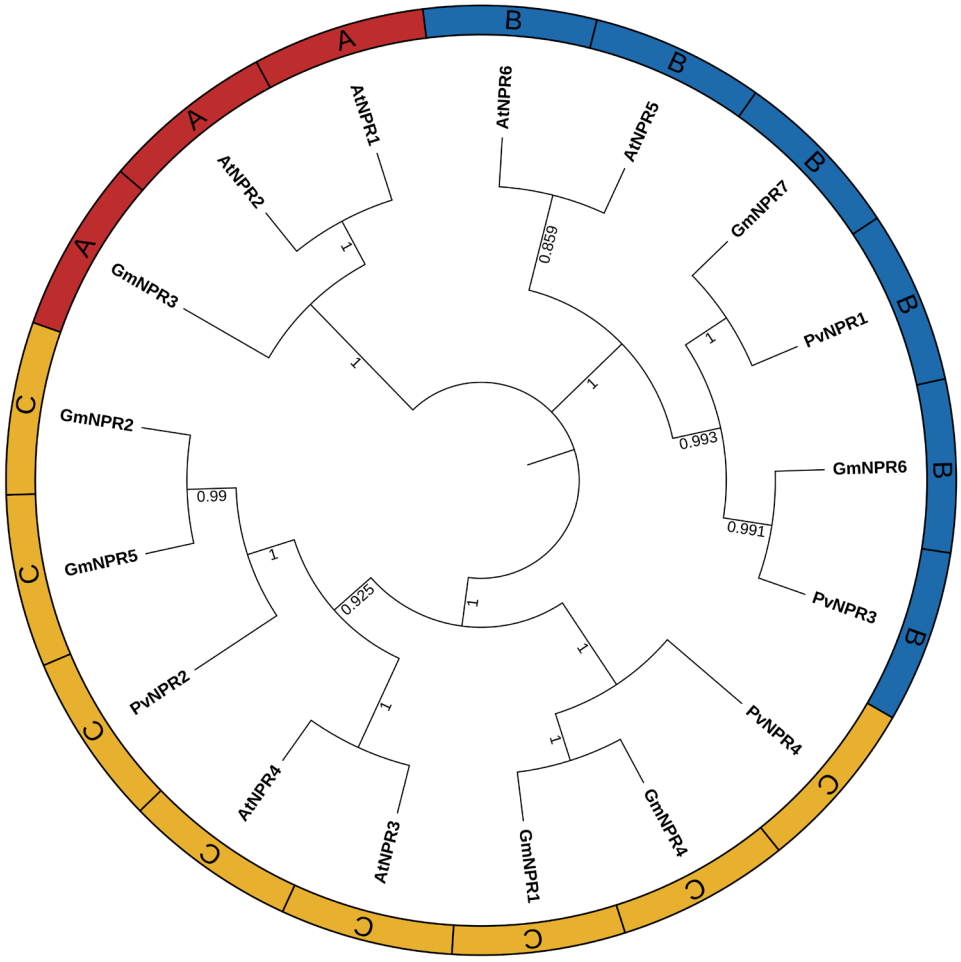
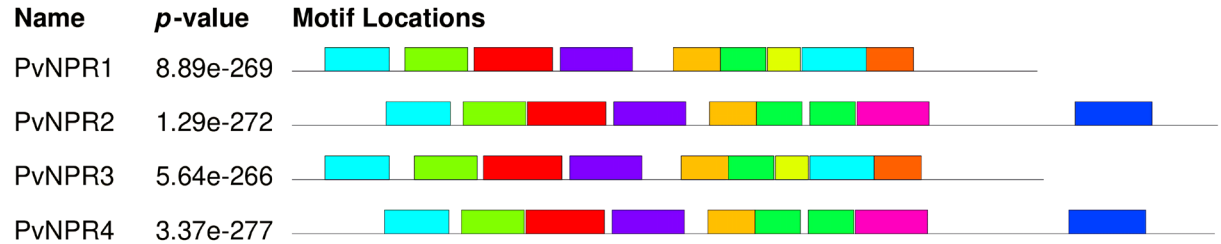


Figure 3. Predicted motifs in *PvNPR-like* genes.

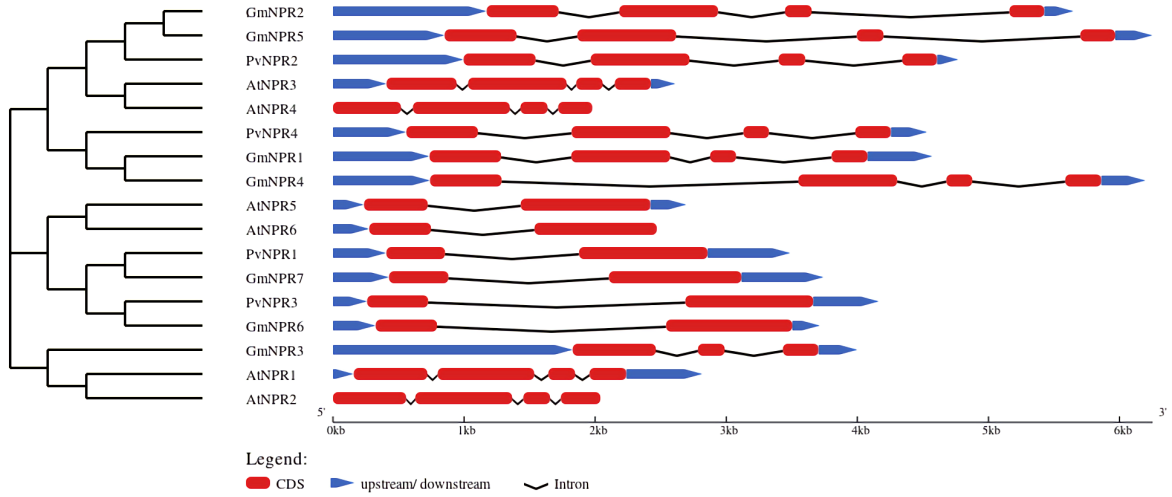


Motif	Symbol	Motif Consensus
1.		CWHDACRPAIDLALDLLYAARYFGVEZLVLLTQKQLLNFVEKALIEDVIP
2.		AAYSDAHIDVEGRTVDAHRCILAARSLFFHELFKGPVPGSG
3.		MSGLIPYGSVGYEVFLJFLQYLYSGQLKIVPQEVEARPNC
4.		RKQEMQQLWSTCIHLVAKSGLPPEVLAKELPHDVVAKIEELRLKSQ
5.		KKIRRIRKALDSDDVELVKLLLNGEGJTLD
6.		EALALHYAAEYCDPEVIKALLGLGAADVB
7.		MKTVENGRRFFPHCSEVLDKFLEDDLPDLFFLEKGSZEEQRIKKARFIE
8.		TFDGQSALSICRRLTRRKDYHEKTEKCKESNKDRJCIDVLEREMRR
9.		HIEPNKLRLCLELVQSAALVLSREEGNNNN
10.		PAGPAGKTPLHIAAEMVSPEM

Table 3. Sequence information of predicted motifs in *PvNPR-like* genes.

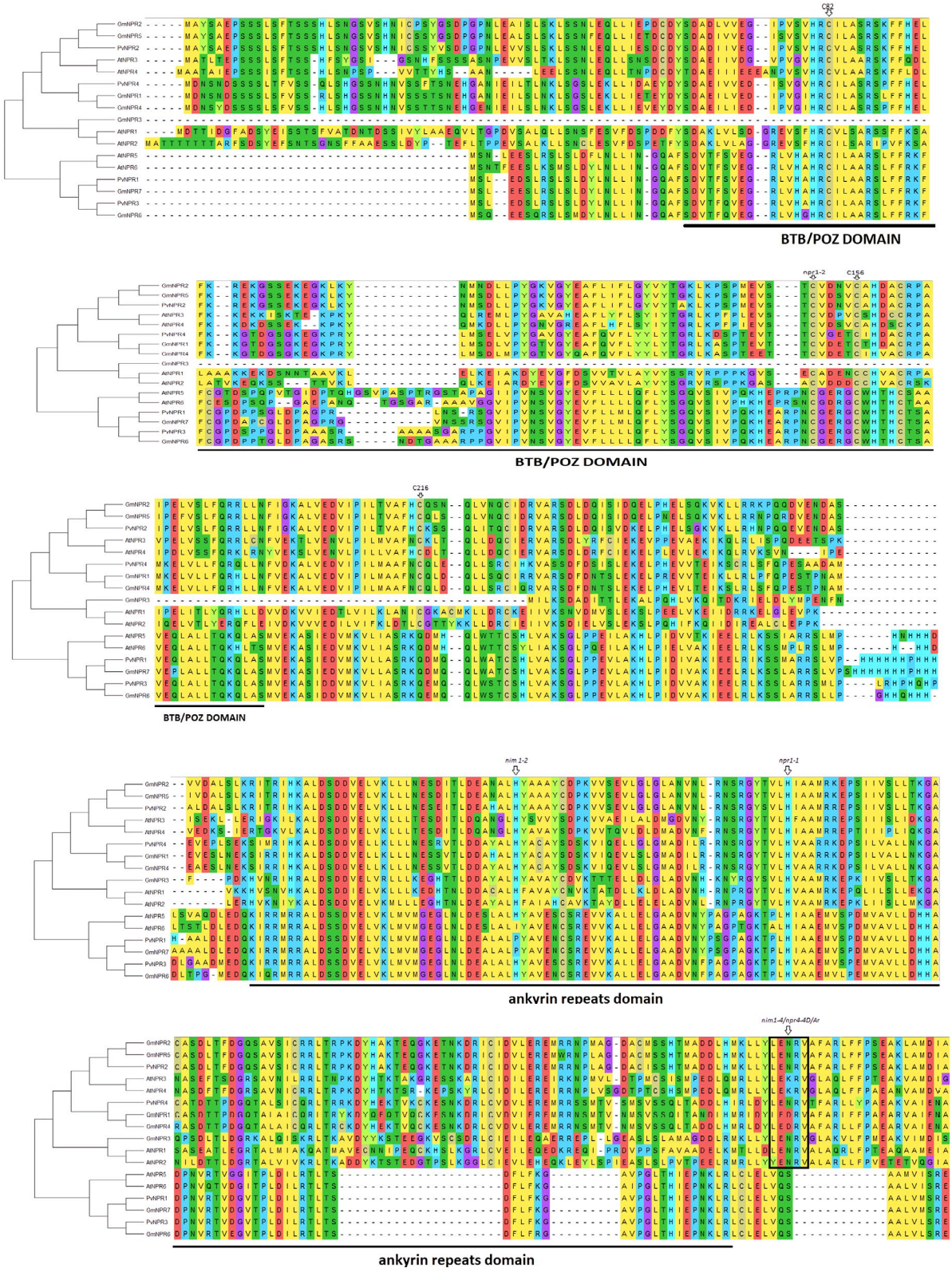
Motif ID	Width	Best Possible Match	Domain
1	50	CWHTHCTPAIDYALDTMYAARYFGIEQLVLLFQKQ LANFVEKASIEDVMP	Regulatory protein NPR5/6
2	41	QVYSDHTFQVEGRTVHGHRCILAARSLFFHFFFKGP TPGSG	N/A
3	40	MSGVIPYGSVGYEVFLIFLQYLYTGQVKIVPQKHET CPNC	Regulatory protein NPR5/6
4	46	FKCQMQQLWSTCIHKVAKSGLPPIVLEKHLPIDIVA KIKELRIKPQ	Regulatory protein NPR5/6
5	30	QKIRRMHKALDSDDVELVKLMVMGEGITLD	N/A
6	29	EYYALHYAAEYCDPKVIKALLGLGACDVN	N/A
7	49	MKTVKMGRRFFPHCSEVLDKFMEDDMPDVFFLEK GTQEEQRMKKTRFME	Regulatory protein NPR
8	46	TFDGQTAVSICQRLTRRKDYHEKTEQCKETNKDRIC IDVLEREMRR	Regulatory protein NPR
9	30	HIEPNKLRLCLELVQSAALVMSREEGNNNN	N/A
10	21	PAGPAGKTPLHIAAEMVSPEM	N/A

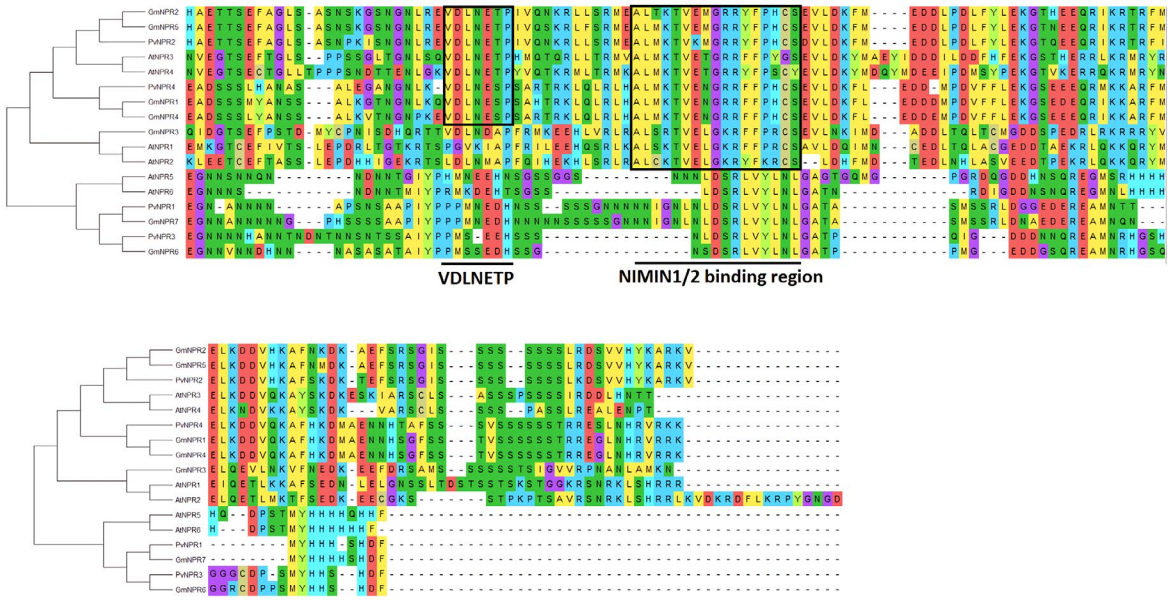
Figure 4. Number, position, and length of inton-exons in *PvNPR-like* genes



P. bretschneideri NPR-like genes were found to have 3 intron numbers and 4 exon numbers. Only one intron region was detected in 2 *PbNPR1* genes (Wei *et al*., 2021). In the *NPR-like* genes of *G. hirsutum*, 3 introns and 4 exons were detected. An intron region was found in the *NPR-like* genes of *G. hirsutum*, which showed homology to *AtNPR5* and *AtNPR6* in the phylogenetic tree. This situation was similarly detected in *P. bretschneideri NPR-like* genes. These observations show that groups homologous to *AtNPR5* and *AtNPR6* have similar intron and exon structure. *NPR-like* proteins in *P. vulgaris, G. max* and *A. thaliana* were aligned at the ClustalW interface. The *BTB/POZ* and *NPR-like*_C domains and ankyrin repeats that should be present in *NPR-like* proteins were identified and marked (Figure 5). In previous studies, it has been reported that *BTB/POZ* and *NPR-like*_C domains and ankyrin repeats are found in *NPR-like* genes of different plant species.

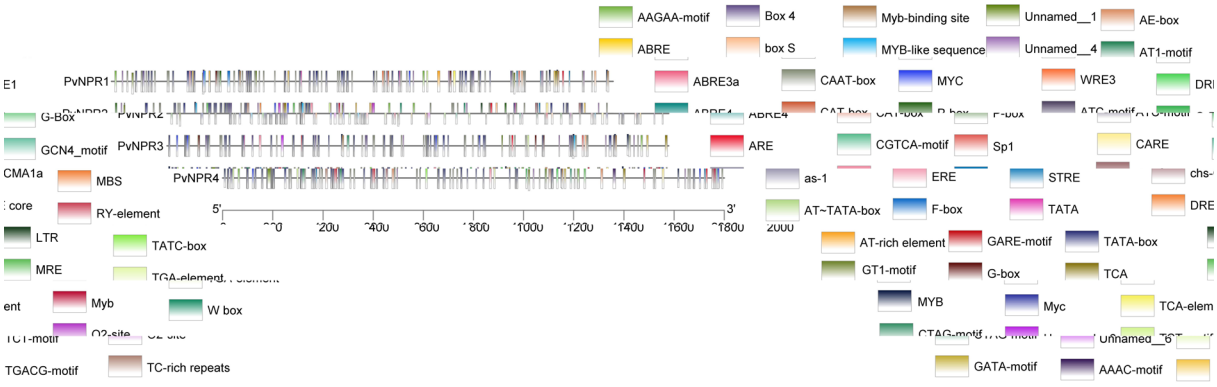
Figure 5. Alignment and conserved domains of *NPR-like* proteins in *P. vulgaris, G. max*, and *A. thaliana*.





The *cis-acting* elements analyzed with the PlantCARE database were plotted using the Tbtools program (Figure 6). As a result of this analysis, it was determined that the elements involved in the stress response are found in these genes (Supplementary File 2). The most abundant elements were determined as following: TATA-box (%42), CAAT-box (%18,8), AT~TATA-box (%3,8), Box 4 (%2,1), MYB (%2,1), ARE (1,6), MYC (1,5) and as-1 (%1). Looking at the stress elements, the most elements were found in *PvNPR3* and *PvNPR4* (18), while the least element was found in *PvNPR3* (11). The most abundant element on a gene was MYB, which was found 5 times on the *PvNPR1, PvNPR3* and *PvNPR4* genes. Similarly, *cis-acting* elements such as TATA-box, CAAT-box, W-box and as-1 were detected in the *NPR* genes of *T. aestivum* (Liu *et al*., 2019). The distribution of stress-related *cis-acting* elements in the *NPR* genes of *P. bretschneider* was investigated. In *PbNPR* genes, the most MBS (13) and MYB (9) *cis-acting* elements were found on one gene.

Figure 6. Distribution of *cis-acting* elements in the 5' upstream region of *PvNPR-like* genes



3.3. Comparative and synteny analysis of *NPR-like* genes in *A. thaliana*, *G. max* and *P. vulgaris*

The synteny map between the *NPR-like* proteins of *A. thaliana*, *G. max*, and *P. vulgaris* was drawn. As a result of synteny analysis, orthology was detected in 9 genes belonging to *P. vulgaris* (Pv) and *G .max* (Gm) (Figure 7A). 4 synteny relationships were found between *P. vulgaris* (Pv) and *A. thaliana* (At) (Figure 7B). In the synteny analysis performed between

wheat species, 6 synteny relationships were found between the D genome of *T. astivum* and *Aegliops tauschii* Coss., and 5 synteny relationships between the A genome of *T. aestivum* and *T. urartu* (Liu *et al*., 2019).

3.4. Gene Ontology Analyzes of *PvNPR-like* Genes

As a result of the gene ontology analyzes performed in the Omics Box database, the processes in which *PvNPR-like* genes are most involved were found as "regulation of salicylic acid-mediated signaling pathway", "salicylic acid-mediated signaling pathway-systemic acquired resistance", "regulation of jasmonic acid-mediated signaling pathway", "jasmonic acid-mediated signaling pathway-induction of systemic resistance" (14.29%) and " fungi-sensitive defense process" (14.28%) (Figure 8).

3.5. Homology Modeling of *PvNPR-like* Proteins

PvNPR-like proteins were screened with the help of blastp in the Protein Data Bank (PDB). As a result of the scan, three-dimensional homology models of these proteins were formed, which were analyzed via the Phyre2 database (Figure 9).

Figure 7. Synteny analysis of *NPR-like* genes of *P. vulgaris* and *G. max* (A), *P. vulgaris* and *A. thaliana* (B)

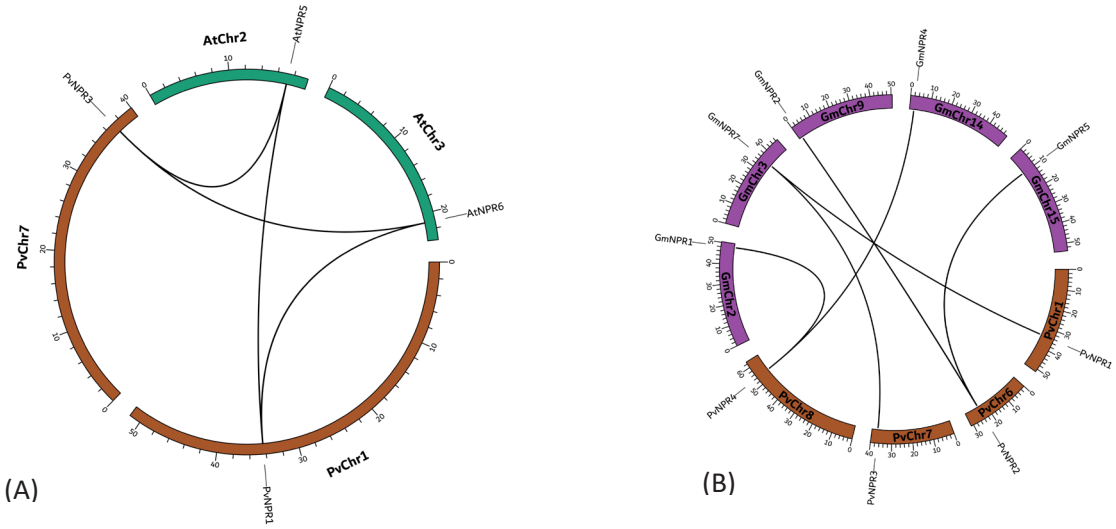


Figure 8. Gene ontology analysis of *PvNPR-like* proteins

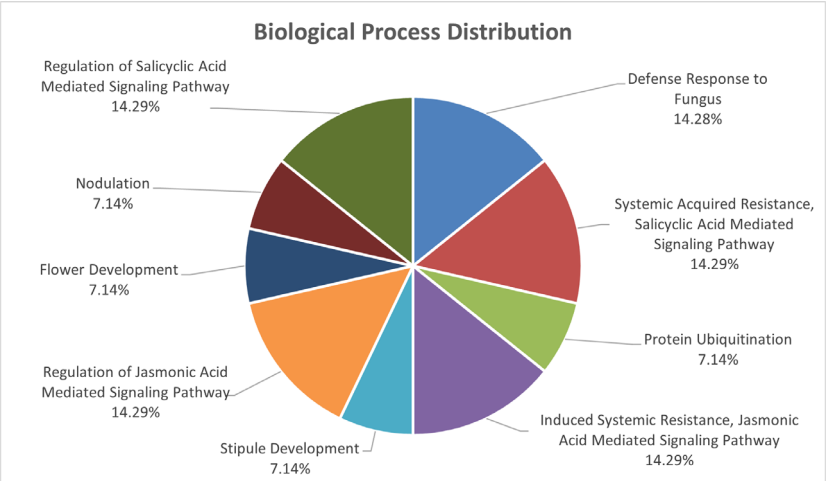

Biological Process Distribution
Regulation of Salicyclic Acid Mediated Signaling Pathway 14.29%
Defense Response to Fungus 14.28%
Nodulation 7.14%
Systemic Acquired Resistance, Salicyclic Acid Mediated Signaling Pathway 14.29%
Flower Development 7.14%
Protein Ubiquitination 7.14%
Regulation of Jasmonic Acid Mediated Signaling Pathway 14.29%
Induced Systemic Resistance, Jasmonic Acid Mediated Signaling Pathway 14.29%
Stipule Development 7.14%

Figure 9. 3D structure modeling of *PvNPR-like* proteins

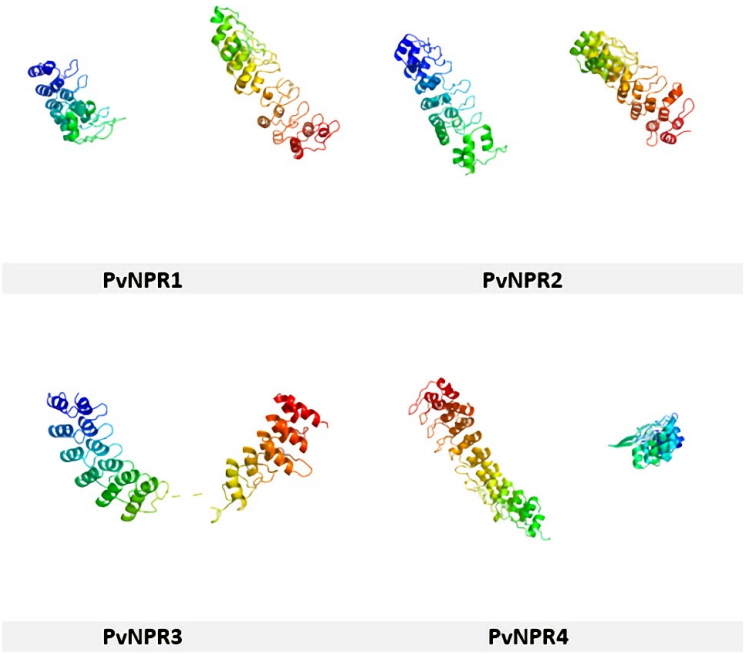


Table 4. Information of other proteins with which *PvNPR-like* genes interact.

Protein ID	Phytozome ID	Related Domain
XP_007162482.1	Phvul.001G156000.1.p	Acetyllysine binding site
XP_007135667.1	Phvul.010G148200.1.p	ATP binding site
XP_007153692.1	Phvul.003G056900.1.p	Hypothetical protein
XP_007136203.1	Phvul.009G026900.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007133721.1	Phvul.011G203400.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007153599.1	Phvul.003G049300.1.p	Cullin protein neddylation domain
XP_007151221.1	Phvul.004G027900.1.p	Cullin protein neddylation domain
XP_007150072.1	Phvul.005G124100.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007163615.1	Phvul.001G249300.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007159742.1	Phvul.002G263400.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007142889.1	Phvul.007G025500.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007141120.1	Phvul.008G169000.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007142625.1	Phvul.007G003600.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007162093.1	Phvul.001G123300.1.p	bZIP domain of bZIP transcription factors: a DNA-binding and dimerization domain
XP_007136488.1	Phvul.009G049400.1.p	ubiquitin-like (Ubl) domain
XP_007152897.1	Phvul.004G169300.1.p	cAMP-binding domain of CRP or a regulatory subunit of cAMP-dependent protein kinases
XP_007146707.1	Phvul.006G062800.1.p	ATPases Associated with a wide variety of cellular Activities superfamily

3.6. *PvNPR-like* Protein-Protein Interactions

Protein-protein interactions of *PvNPR-like* proteins were taken from the STRING database and shaped with the Cytoscape program (Figure 10). As a result of the analysis, it was found that *PvNPR1* and *PvNPR2* was associated with the bZIP domain of the bZIP transcription factor (9 different associations). In addition, the relationship of this gene with ubiquitin-like domain and a regulatory subunit of cAMP-dependent protein kinases has been determined. *PvNPR2* was found to be associated with the Cullin protein neddylation domain. The interactions of *PvNPR3* were identical with *PvNPR1*, except for a regulatory subunit of cAMP-dependent protein kinases. On the other hand, *PvNPR4* was found to be associated with the bZIP domain of the bZIP transcription factor and the ATP binding site (Table 4). The ankyrin-repeat domain interacts with the TGA of the bZIP transcription factor, and the BTB/POZ domain helps NPR1 dimerize (Wei *et al*., 2021).

3.7. Expression Levels of *PvNPR-like* Genes Under Salt and Drought Stress

Expression profiles of *PvNPR-like* genes under drought and salt stress were analyzed in specialized tissue libraries. Considering the expression levels, significant differences were observed in the expression level of *PvNPR3* under both drought and salt stress. Expression level of *PvNPR3* and *PvNPR1* increased in drought stress. No significant changes were observed in other genes. In salt stress, the expression level of *PvNPR3* decreased while the expression level of *PvNPR4* increased. No significant changes were observed in other genes (Figure 11). Heat map analysis of *G. hirsutum NPR* genes revealed differences in expression levels of *GhNPR* genes in different tissues of the plant (Agarwal *et al*., 2020).

Figure 10. Protein-protein interaction of *PvNPR-like* genes

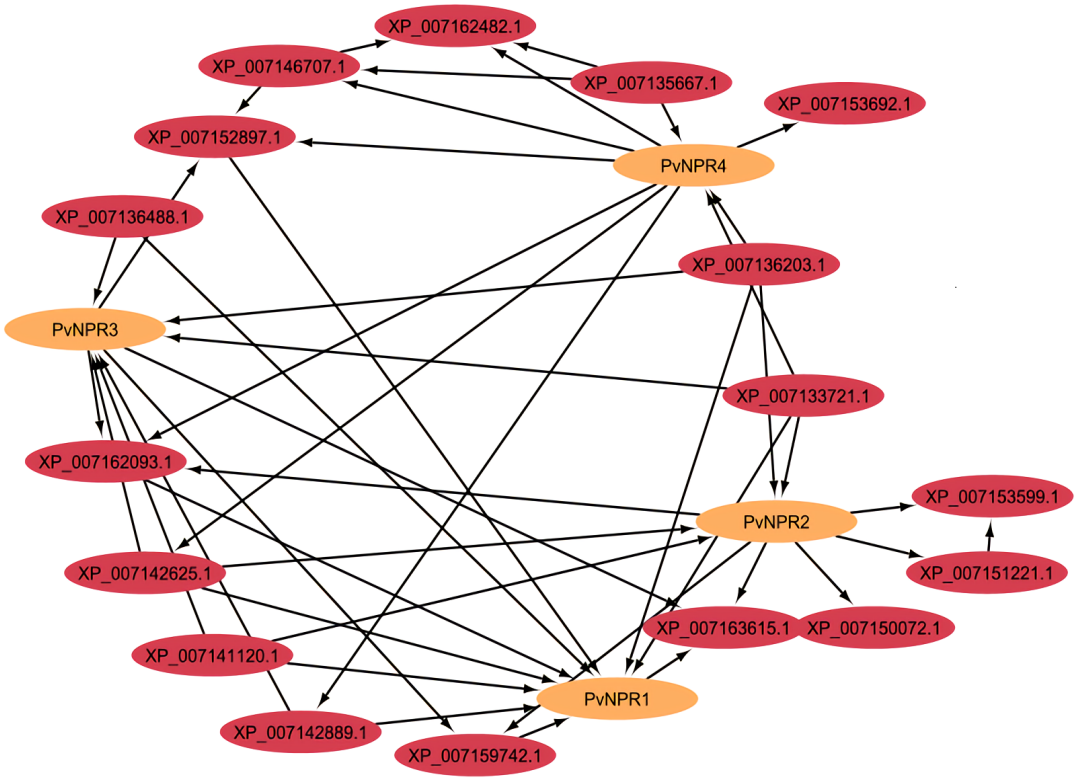
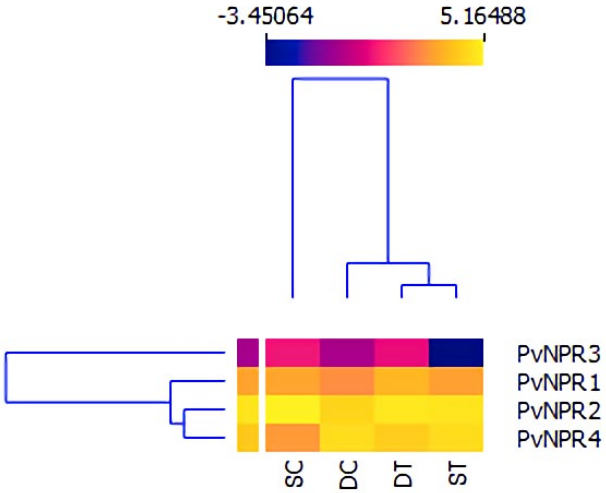


Figure 11. *In silico* gene expression analysis of *PvNPR-like* genes under salt and drought stress (SC: Salt Control, ST: Salt Treatment, DC: Drought Control, DT: Drought Treatment).



4. CONCLUSION

As a result of in silico analysis with the *PvNPR-like* gene family in the *P.vulgaris* genome, four *PvNPR* members were identified. The detected genes were distributed on four different chromosomes. *NPR-like* genes in *Phaseolus vulgaris*, *Glycine max* and *Arabidopsis thaliana* plants are classified into 3 different groups. As a result of conserved motif analysis, 6 different unique motifs were detected in all *PvNPR-like* genes. As a result of the *cis-acting* element analysis, TGA element was detected on *PvNPR4*. In addition, the interaction of all *PvNPR-like* genes with the bZIP domain supported this relationship. *PvNPR-like* genes have been confirmed to be involved in the salicylic and jasmonic acid signaling pathway. Significant differences were observed among *PvNPR-like* gene family members, especially in *PvNPR3*, in gene expression analyzes of *P.vulgaris* plant under drought and salt stress. In the light of the findings obtained as a result of this study, it will help to elucidate the function of *PvNPR-like* genes in metabolic and physiological pathways.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Burak Muhammed Oner: Writing, Bioinformatics analysis. **Emre Ilhan:** Editing, Validation, Supervision. **Ayse Gul Kasapoglu:** Writing, Visualization. **Selman Muslu:** Writing, Editing. **Ahmed Sidar Aygoren:** Writing, Editing. **Sumeyra Ucar:** Writing, Editing. **Esra Yaprak:** Writing, Editing. **Murat Isiyel:** Writing, Editing. **Recep Aydinyurt:** Writing, Editing. **Murat Aydin:** Editing, Validation, Supervision.

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Apoptotic and Anti-Metastatic Effect of Carvacrol in PANC-1 Human Pancreatic Cancer Cells

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PANC-1 cells

Abstract

Pancreatic cancer is known to have one of the highest mortality rates among other cancer. Monoterpenes are natural compounds obtained from essential oils of plants. Carvacrol is a monoterpene phenolic derived from essential oils of various aromatic plants. In this study, the effects of carvacrol on proliferation, apoptosis and metastasis of PANC-1 human pancreatic cancer cells were investigated. XTT assay was used to determine cell proliferation and viability. mRNA level expression levels of BAX, BCL2, CASP3, CASP7, CASP8, CASP9, CYCS, FADD, FAS and P53 were evaluated for determining the effect of carvacrol on apoptosis by qRT-PCR analysis. In addition, gene expressions of CDH1, CDH2, TIMP1, TIMP2, TIMP3, ZEB1 and ZEB2 were analyzed to evaluate its effect on metastasis. Carvacrol inhibited the PANC-1 pancreatic cancer cell proliferation in dose and time dependent manner. Carvacrol treatment induced apoptosis by changing the expressions of genes important in apoptosis. Moreover, it suppressed the metastasis by effecting CDH1, CDH2, TIMP2, TIMP3 and ZEB2 expressions. In conclusion, carvacrol showed apoptotic and anti-metastatic effects in PANC-1 human pancreatic cancer cells. It is thereby concluded that carvacrol may have a therapeutic potential in pancreatic cancer.

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1. INTRODUCTION

Pancreatic cancer ranks seventh in cancer-related deaths in the worldwide (Sung *et al*., 2021). It is estimated that 62,210 new cases will be diagnosed and 49,830 patients are expected to die from the pancreatic cancer in the United States in 2022. The incidence of this disease has been increasing by approximately 1% per year. Mortality rates from pancreatic cancer have increased by 0.2% each year in recent years (American Cancer Society, 2022).

High toxicity in cancer treatment may affect the specificity of the treatment. For this reason, the search for non-toxic treatment methods for normal cells continues. Therefore, it has been observed that natural compounds can be used clinically and interest in the use of these compounds in medicine has increased. Monoterpenes are natural compounds obtained from essential oils of plants (Rejhová *et al*., 2018). One of these, carvacrol (5-isopropyl-2-methylphenol) is a natural bioactive monoterpenoid phenol derived from some aromatic plant essential oils of Lamiaceae family (Baj *et al*., 2020). Studies have demonstrated that this natural compound have anticancer (Li *et al*., 2021), gastroprotective (Souza *et al*., 2017), anti-inflammatory (Guimarães *et al*., 2012), antibacterial (Ben Arfa *et al*., 2006), antioxidant (Guimarães *et al*., 2010) and analgesic (Can Baser, 2008) effects. In addition, it was reported

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that carvacrol showed antiproliferative effect in many cancer cells including prostate (Luo *et al*., 2016), stomach (Günes-Bayir *et al*., 2018a), colon (Fan *et al*., 2015), liver (Yin *et al*., 2012) and lung (Koparal and Zeytinoglu, 2003) cancer cells. The anticancer effect of carvacrol however has not been fully elucidated.

Previously, it was shown that carvacrol suppressed cell viability of human prostate cancer PC-3 cell line in a dose and time dependent manner. Moreover, it induced apoptosis by modulating BAX, BCL2 and caspase (CASP) activity (Khan *et al*., 2019). Khan *et al*. (2018a) reported that carvacrol nanoemulsion caused apoptosis induction by elevating BAX, cytochrome c (CYCS), CASP3 and CASP9 by decreasing BCL2 in doxorubicin resistant human lung adenocarcinoma A549 cells. Previously, it was stated that carvacrol had an apoptotic effect by elevating BAX level and downregulating BCL2 expression in U2OS and 143B human osteosarcoma cells. Moreover, carvacrol suppressed MMP9 expression, invasion and migration in the same study (Zhang *et al*., 2021). Liang *et al*. (2022) showed that carvacrol significantly decreased leukemia cell viability. In a study, it was reported that carvacrol could contribute to human Jurkat T cell activity modulation by suppressing IL-2 and IFN-γ levels and also NFAT-2 and AP-1 transcription factors (Gholijani *et al*., 2015). In a previous study, MCF-7, MDA-MB-453, MDA-MB-231, BT-474 and BT-483 breast cancer cells were treated with 10-500 µM carvacrol for 24 h. CCK-8 cell viability assay results showed that carvacrol between 50 and 500 µM dose-dependently suppresses cell viability in all cells. In the same study, it was shown that 200 µM carvacrol treatment caused cell cycle arrest by regulating some cyclin proteins in MDA-MB-231 cells (Li *et al*., 2021). In this study, we aimed to investigate possible effect of carvacrol on cell proliferation and the expression of apoptosis and metastasis related genes in PANC-1 human pancreatic cancer cells.

2. MATERIAL and METHODS

2.1. Chemicals

Carvacrol was commercially purchased from Tokyo Chemical Industry (TCI). XTT kit and FBS were obtained from Biological Industries. DMEM, penicillin/streptomycin and PBS purchased from Gibco. QIAzol and cDNA synthesis kits were obtained from Qiagen and Bio-Rad, respectively.

2.2. Cell Culture

PANC-1 human pancreatic cancer cells (ATCC®CRL-1469™) were obtained from the American Type Culture Collection (USA). PANC-1 cells were cultured in DMEM medium containing 1% penicillin/streptomycin, 10% FBS and 2 mM L-glutamine, and proliferated in an incubator at 37ºC, 5% CO_2 and humidified 95% air.

2.3. Cell Viability Assay

PANC-1 cells were treated to prepared carvacrol solutions for 25, 50, 100, 200, 500 and 1000 µM for 24, 48 and 72 h. Carvacrol was dissolved in ethanol. The XTT method was used to evaluate the cytotoxic effect of carvacrol on PANC-1 cells. XTT test was performed according to the protocol in our previous study (Secme *et al*., 2018). IC_{50} value of carvacrol in PANC-1 cells was determined by calculating with the CompuSyn Version 1.0 software.

2.4. Total RNA Isolation, cDNA Synthesis and qRT-PCR Analysis

Expressions of important genes in apoptosis (BAX, BCL2, CASP3, CASP7, CASP8, CASP9, CYCS, FADD, FAS and P53) and metastasis (CDH1, CDH2, TIMP1, TIMP2, TIMP3, ZEB1 and ZEB2) pathways were evaluated after total RNA isolation and cDNA synthesis. ACTB was used as housekeeping gene and internal control. Expressions of these genes were

determined by qRT-PCR analysis using the primers and the protocol described in elsewhere (Eroğlu-Güneş *et al*., 2021).

2.5. Statistical Analysis

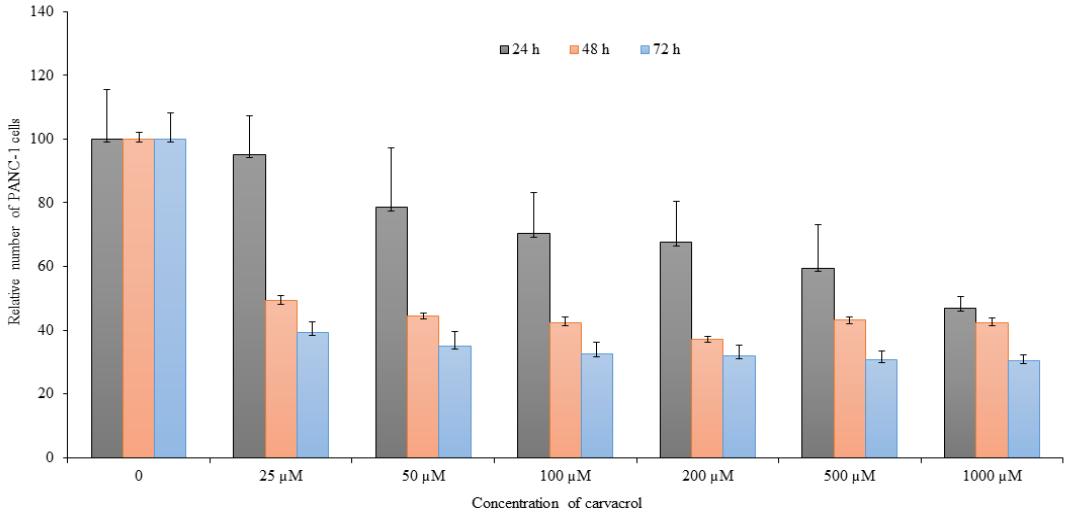
After three technical replicates, Ct values were obtained and normalized using the reference gene and the data were shown as mean ± SD. The qRT-PCR analysis was conducted with the $2^{(-\Delta Ct)}$ method. Experimental groups were compared using the independent samples *t-test* in SPSS 26.0 statistical analysis program. $p < 0.05$ was considered as statistically significant.

3. RESULTS and DISCUSSION

3.1. Cytotoxic Effect of Carvacrol in PANC-1 Cells

Cytotoxic effect of carvacrol in PANC-1 was determined by XTT assay. Carvacrol inhibited the cell proliferation of pancreatic cancer cells time and dose dependent manner. The half maximal inhibitory concentration (IC_{50}) dose of carvacrol in PANC-1 cells was found to be 664.02 µM for 24 h using CompuSyn version 1.0 software (Figure 1).

Figure 1. Effect of carvacrol on the PANC-1 cell viability. IC_{50} dose of carvacrol in PANC-1 cells was found to be 664.02 µM.



Apoptosis is an important cell death mechanism in cancer therapy. Anti-apoptotic BCL2, pro-apoptotic BAX, apoptotic proteins including CASP3, CASP7, CASP8, CASP9, CYCS, FADD and FAS play a critical role in regulation of programmed cell death. P53, known as a tumor suppressor protein and transcription factor, regulates the expression of several genes, including the cell cycle and apoptosis (Aubrey *et al*., 2018).

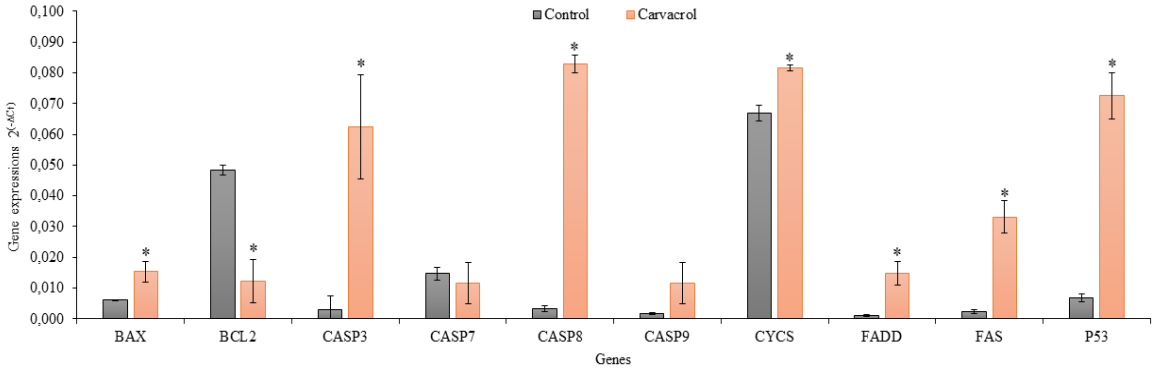
Essential oils obtained from aromatic plants are known to have strong anticancer effects. In a previous study, it was shown that carvacrol suppressed cell viability in dose and time dependent manner in human prostate cancer DU145 cells. In the same study, it was reported that the IC_{50} dose of carvacrol in these cells was 84.39 µM for 24 h and 42.39 µM for 48 h (Khan *et al*., 2017). Günes-Bayir *et al*. (2018b) showed that IC_{50} value of carvacrol was 82.57 µM for 24 h in gastric adenocarcinoma AGS cells. In addition, carvacrol has been reported to induce cytotoxicity, apoptosis and increased reactive oxygen species in these cells. In a previous study, effects of carvacrol in eight liver hepatocellular carcinoma cell lines were evaluated. Carvacrol suppressed cell viability of all these cells between 10-300 µM. Hep-G2 was the most

sensitive cell with inhibition rate 90% at 300 µM. SNU-182 and SNU-389 were least sensitive cells with 40% inhibition rate at 300 µM (Yin *et al*., 2022). In another previous study (Heidarian and Keloushadi, 2019), IC_{50} dose of carvacrol was 360 µM in PC3 human prostate cancer cells. Fan *et al*. (2015) reported that IC_{50} doses of carvacrol were 544.4 and 530.2 µM in HCT116 and LoVo human colon cancer cells, respectively. Also, the cell viability of HeLa human cervical cancer cells was suppressed with carvacrol treatment at IC_{50} dose of 556 µM (Potočnjak *et al*., 2018). In a study investigating the effect of carvacrol on ovarian cancer, it was reported that its IC_{50} value in SKOV-3 ovarian cancer cells was 322.50 µM at 24 h and 289.54 µM at 48 h (Elbe *et al*., 2020).

3.2. Expression of Apoptosis Related Genes in Carvacrol Treated PANC-1 Cells

Expression changes of genes in apoptosis pathway were evaluated by using qRT-PCR in carvacrol treated PANC-1 cells. Carvacrol caused a significant increase in BAX, CASP3, CASP8, CYCS, FADD, FAS and P53 but a decrease in BCL2 expression. Moreover, it was also observed that it insignificantly downregulated CASP7 expression but elevated CASP9 expression (Figure 2).

Figure 2. Effect of carvacrol on apoptosis genes in PANC-1 cells. (*, $p<0.05$)

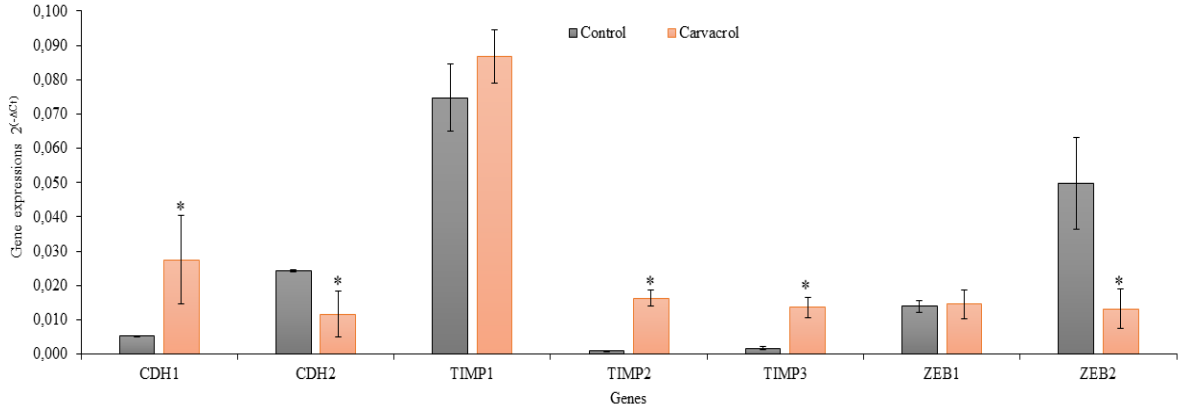


One of the most important points in the occurrence of cancer is the disruption of the balance between cell proliferation and apoptosis. Pancreatic cancer is treated with standard treatment options that cause cell death including chemotherapy and radiotherapy. However, the side effects experienced in the treatments limit the therapeutic applications. For pancreatic cancer therapy, it is important to discover novel agents that specifically target cancer cells and are less toxic to normal cells. Bhakkiyalakshmi *et al*. (2016) showed that carvacrol induced apoptosis in HL-60 human acute promyelocytic leukemia and Jurkat human acute T cell leukemia cells. The IC_{50} value of carvacrol was reported to be 100 µM for HL-60 cells and 50 µM for Jurkat cells. It was stated that BAX and CASP3 expressions were increased, however BCL2 expression was downregulated in carvacrol treated cells (Bhakkiyalakshmi *et al*., 2016). Khan *et al*. (2018b) reported that carvacrol nanoemulsion caused apoptosis by effecting proteins associated with apoptosis including BAX, BCL2, CYCS, CASP3 and CASP9 in A549 human lung cancer cells. Carvacrol also suppressed proliferation of cervical cancer HeLa cells via caspase-dependent apoptosis (Ahmad and Ansari., 2021). In a previous study, 300 µM of carvacrol significantly induced population of apoptosis in both JAR and JEG3 choriocarcinoma cells compared with nontreated cells by activating expression of BAX, BAK and CYCS (Lim *et al*., 2019).

3.3. Expression of Metastasis Related Genes in Carvacrol Treated PANC-1 Cells

In order to determine the effects of carvacrol on metastasis, changes in CDH1, CDH2, TIMP1, TIMP2, TIMP3, ZEB1 and ZEB2 gene expression were evaluated by qRT-PCR. The results showed that carvacrol significantly upregulated CDH1, TIMP2 and TIMP3 expressions. It also significantly reduced CDH2 and ZEB2 expressions. No significant changes were observed in ZEB1 expression (Figure 3).

Figure 3. Effect of carvacrol on metastasis genes in PANC-1 cells. (*, p<0.05)



Approximately 80% of pancreatic cancer patients are diagnosed after metastasis and disease progression due to the late onset of symptoms (Kuehn, 2020). EMT (Epithelial mesenchymal transition) is an important step in the formation of invasion and metastasis. In the EMT process, epithelial cells lose their epithelial properties by proteins altering the expression of cell-cell junction proteins. For example, E-cadherin was downregulated and initiate expression of mesenchymal-related proteins including N-cadherin (Hay and Zuk, 1995). In addition, ZEB1 and ZEB2 transcription factors are considered as the main transcriptional regulators of epithelial mesenchymal transition in this process (Peinado *et al*., 2007). Carvacrol application resulted in an elevated E-cadherin (CDH1) level and a downregulation in N-cadherin (CDH2) and vimentin expression on oral squamous cell carcinoma CAL-27 cells in a previous study (Liu *et al*., 2021). Heidarian and Keloushadi (2019) reported that carvacrol significantly reduced invasion in dose dependent manner in PC3 human prostate cancer cells. In a study, 500 µM doses of carvacrol significantly reduced expression of MMP2 protein in PC-3 and DU145 prostate cancer cells (Luo *et al*., 2016). Chen *et al*. (2015) stated that 561.3 µM IC_{50} dose for 24 h of carvacrol suppressed the proliferation, migration and invasion in U87 glioblastoma cells. In a previous study, carvacrol significantly suppressed invasion and migration in human oral squamous cell carcinoma Tca-8113 and SCC-25 cells by inhibiting MMP2, MMP9, ZEB1 and β-catenin expressions (Dai *et al*., 2016). It was shown that carvacrol suppressed cell invasion by decreasing MMP2 and MMP9 expression levels in HCT116 and LoVo human colon cancer cells (Fan *et al*., 2015). The results of the present study showed that increased E-cadherin (CDH1) and decreased N-cadherin (CDH2) and ZEB2 expression may cause suppression of EMT, an important process in metastasis, by carvacrol. Moreover, it showed antimetastatic effect by reducing TIMP2 and TIMP3 expressions, which play an important role in metastasis.

4. CONCLUSION

In this study, apoptotic and anti-metastatic effects of carvacrol were investigated in PANC-1 human pancreatic cancer cells. Carvacrol stimulated apoptosis by effecting expression levels of BAX, BCL2, CASP3, CASP8, CYCS, FADD, FAS and P53 genes. Moreover, it showed anti-metastatic effect by changing gene expressions of CDH1, CDH2, TIMP2, TIMP3 and ZEB2. In conclusion, further studies are needed to determine whether carvacrol can be used as an anti-cancer agent in the treatment of pancreatic cancer because of its apoptotic and anti-metastatic effect on PANC-1 cells.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Canan Eroglu Gunes: Study design, conducting experiments, statistical analyzes, evaluation of the results, preparation of the manuscript. **Mucahit Secme:** Study design, conducting experiments, statistical analyzes, preparation of the manuscript. **Ercan Kurar:** Statistical analyzes, preparation of the manuscript. **Huseyin Donmez:** Study design, statistical analyzes, preparation of the manuscript.

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The Impact of Cold Acclimatization on Antioxidant Enzyme Activity of *Vicia sativa* L.

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Abstract

Vicia sativa L. is one of most significant forages all over the world, but yield is decreased by cold stress. This study aimed to investigate the cold stress mechanism of *V. sativa* under tissue culture in response to some biochemical analysis. In this study, six *V. sativa* cultivars (Tarım Beyazı, Ankara Moru, 24 Çilli, Kansur and Aygün) were carried out to determine cold resistance *in vitro* conditions. Doğu Beyazı cultivar was used as a cold resistant plant. For the cold acclimation, two weeks old seedlings were incubated in the test chamber set at 4°C for 14 days to induce cold stress. Leaf samples were obtained at 14 days after cold treatment for physiological analysis evaluation. Cold resistant cultivars were chosen using values of thermal degrees. Moreover, antioxidant enzyme activities were determined at cold acclimated and non-acclimated seedlings. Peroxidase enzyme activities gradually increased at cold acclimation compared to those seedlings at non-acclimated. The highest POX activity was found at Ankara Moru seedlings, while the lowest activity was found at Aygün seedlings. SOD and APX activities were detected inversely at cold acclimation compared to those seedlings at non-acclimated. ChlorophyII A, chlorophyII B and carotenoid values were also determined at cold acclimated seedlings. The highest activities and pigment values were detected at Tarım Beyazı seedlings. Whereas, the lowest activities were found at Ankara Moru and Aygün cultivars. Our results displayed that cold acclimation linked to SOD, APX and POX activities and pigment estimation at in vitro conditions.

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1. INTRODUCTION

Agricultural production is significantly decreased by biotic and abiotic environmental factors. Cold stress is an abiotic stress that causes limiting crop cultivation and directly influences plant growth and yield. Crop reductions caused by cold stress are becoming a serious problem in North regions of the world (Mutlu *et al*., 2013; Turk *et al*., 2014). Many plant species are affected by these stress factors, therefore it is very important to improve cold resistant lines. Cold stress induces tissue damage, osmotic imbalance, cell membrane, antioxidant enzyme activities, photosynthesis and the amount of carbon production. As a result of this, crop productivity is decreased (Kovács *et al*., 2011). Plants block the stress damage through various techniques in the development medium. Adaptation to cold stress dissimilar between plant species throughout their growth period and are firstly detected in the phenological properties in the life cycle of development process (Tasgin *et al*., 2003; Tasgin *et al*., 2006). In addition, cold stress participates in changes in various physiological and biochemical pathways, adheres to

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impact and degree of the stress and finally limits plant development. Degree of the cold damage in crops is determined by reduced ionic content, decreased cell divisions, stomatal disorders, and differentiation. Excessive low temperatures are restricted water flow by the crops, influencing ionic instability leading to membrane injury and tissue damage. Acclimation is especially important in crops exposed to extreme temperature of cold during seasons of metabolic activities or growth process (Levitt, 1972). *Vicia sativa* L. is a widely cultivated plant for livestock producers of various agricultural areas because of its superior feeding rate. *V. sativa* is grown for its great yield and potential for high input nitrogen, N fixation, soil conservation quality as well as its role on wide pest resistance, and nutritional feeding. *V. sativa* is considered sensitive to cold. However, its productivity could be greatly affected by the cold stress and also its reduced quality of crops (Maqbool *et al*., 2010; Siddiqui, 2015). Therefore, development of *V. sativa* cultivars greatly resistant to cold stress is rapidly required. Even though traditional breeding methods have provided reasonable improvement eliminating cold stress, these techniques currently have some limitations, because low temperature tolerance is controlled by quantitative traits and participates in multiple biochemical and molecular mechanisms (Zhou *et al*., 2018). The current report was performed to define the cold resistance of different *V. sativa* cultivars under in vitro conditions and to detect the relationship of changes in enzymatic activities to cold tolerance in *V. sativa* seedlings.

2. MATERIAL and METHODS

2.1. Plant Material and Cold Acclimation Treatment

V. sativa seeds were obtained from Seed Gen Banc Field Crops Research Center, Ankara, Turkey. Prior to experiments, seeds were disinfected for 15 min with water/bleach (10:1, commercial NaOCl) solution and then sterilized three times with ddH_2O. Seeds were transferred in hormone free MS medium. Six *V. sativa* and control seedlings from cultivars were carried out to determine the cold tolerance. For cold acclimation, 14-day-old seedlings were incubated in a growth chamber set at 4°C to induce cold treatment for 14 days, with light condition (day/night 16/8 h photoperiod, 50µmol m) and relative humidity (65%). Leaf samples were obtained at 14 days after cold treatment for SOD, APX and POX activity and pigment estimation evaluation.

2.2. Antioxidant Enzyme Activities

Six cold acclimated (Tarım Beyazı, 24 Çilli, Ankara Moru, Aygün and Kansur one (Doğu Beyazı-cold resistant cultivar) non acclimated cultivars were carry out to determine the antioxidant enzyme activities. Leaf samples (0.5 g) were crushed with a mortar and homogenized in ice-cold 0.2M phosphate buffer (pH 7.0) containing 0.1M methylenedinitrilotetra acetic acid (EDTA). Extract was centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was utilized to measure the activity of APX and SOD.

APX was analyzed by recording the decrease in absorbance at 290 nm in 3 mL sample mixture including 50mM potassium phosphate buffer (pH 7.0), 0.5mM sodium ascorbate, 0,1mM EDTA, 0.2 mL supernatant (Asada, 1981).

SOD activity was determined by monitoring the reduction in absorbance of nitro- blue tetrazolium (NBT) dye (Dhindsa et al., 1981). Sample mixture included 13mM methionine, 2µM riboflavin, 75µM NBT, 0.1mM EDTA, 50mM sodium carbonate, 50mM phosphate buffer (pH 7.8), and 0.1 mL of the extract. Riboflavin was added at the end and the tubes were shaken and placed 30 cm below a light bank containing two fluorescent tubes. After 20 min, the reaction was finished by covering the tubes with a black cloth. The absorbance was recorded spectrophotometrically at 560 nm.

The POX activity was measured by monitoring the increase in absorbance at 470 nm in 50mM phosphate buffer (pH 5.5) containing 1mM guaiacol and 0.5 mM H_2O_2 (Siddiqui *et al*., 2015).

2.3. Pigment Estimation

Seedling samples were isolated 80% acetone and obtained at 645 and 663 nm were determined chlorophyll A, chlorophyll B and total carotenoid amounts were then measured.

2.4. Statistical Analysis

Each experiment was repeated at three times. Analysis of variance was conducted using one- way ANOVA test using SPSS 13.0 and means were compared by Duncan test at the 0.05 level of confidence.

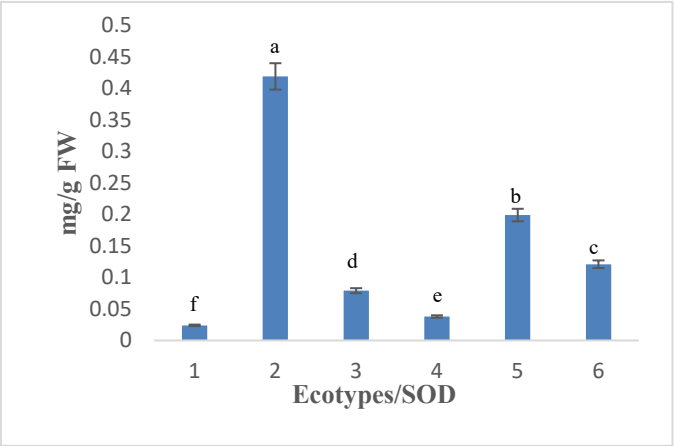
3. RESULTS and DISCUSSION

V. sativa is particularly susceptible to cold stress during seed germination, vegetative growth and flowering stages (Zhou *et al*., 2018). Cold stress in *V. sativa* is largely linked to the early germination phase. Therefore, selection and improvement of cold-resistance and remarkably productive varieties is needed to facilitate the increased *V. sativa* grown in Turkey. In present study, 6 cultivars of *V. sativa* were subjected to cold acclimation. As shown in Figures, all tested cultivars indicated various activities under normal and extreme cold conditions. Moreover, antioxidant enzyme activities displayed by cultivar Tarım Beyazı under cold acclimation showed that this cultivar had sufficient extent of cold resistance in the aspect of leaves. Improvement of cold tolerance in plants is remarkably linked to the enrichment of antioxidant enzyme activity. Several species have been well documented in terms of cold stress (Zhou and Guo, 2005). Earlier reports have indicated that antioxidant activities and chlorophyll content in *V. sativa* and *alfalfa* is remarkably associated with extreme cold resistance (Castonguay *et al*.,1995; Bertrand *et al*., 2017; Bezirganoglu *et al*., 2018). SOD activity was significantly different between *V. sativa* cultivars in cold acclimation and non-acclimated. The results of SOD activities alters between 0.024 and 0.419 nmol g^{-1} FW. The highest SOD value was found in 'Tarım Beyazı' (0.419 nmol g^{-1} FW) followed by '24 Çilli' (0.199 nmol g^{-1} FW), 'Aygün' (0.121 nmol g^{-1} FW). Whereas, the lowest was observed in the Ankara Moru (0.079 nmol g^{-1} FW) and Kansur (0.038 nmol g^{-1} FW) cultivars (Figure 1, Table 1). Peroxidase increased in seedlings of tested cultivar under cold acclimation. A continuous increase in peroxidase was determined in + 4 ^{0}C acclimated in all cultivars. Significant differences were observed between ecotypes compared to control. The highest peroxidase value was found in '24 Çilli' (1.191 nmol g^{-1} FW) whereas, the lowest value was found in Tarım Beyazı (0.126 nmol g^{-1} FW) (Figure 2, Table 1). APX value was also different between tested seedlings in both cold acclimation and non-acclimated conditions. The results of the APX analyzes ranged between 0.017 nmol g^{-1} FW and 3.100 nmol g^{-1} FW. Significant differences were observed between ecotypes compared to control. The lowest APX amount was found in 'Kansur' (0.017 nmol g^{-1} FW), followed by 'Aygün' (0.099 nmol g^{-1} FW), 'Ankara Moru' (0.170 nmol g^{-1} FW) (Figure 3, Table 1).

Table 1. Changes in SOD, APX and POD activity of six *V. sativa* cultivars.

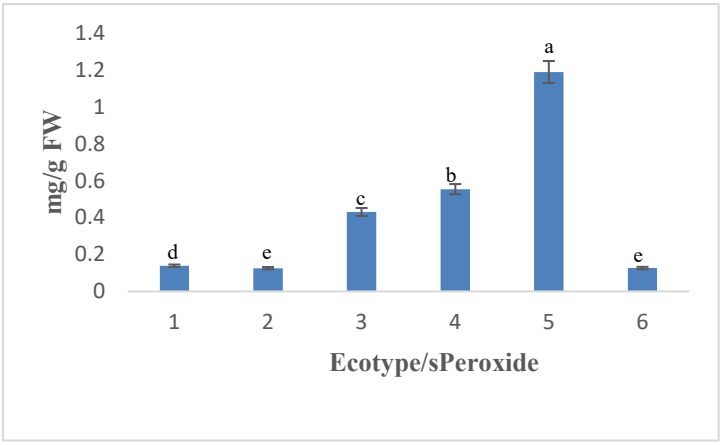
Ecotypes	SOD	APX	POD
Doğu Beyazı	0.024±0.001^{f}	1.237±0.166^{b}	0.139±0.001^{d}
Tarım Beyazı	0.419±0.001^{a}	3.100±1.000^{a}	0.126±0.001^{e}
Ankara Moru	0.079±0.001^{d}	0.170±0.001^{d}	0.431±0.001^{c}
Kansur	0.038±0.001^{e}	0.017±0.001^{d}	0.555±0.001^{b}
24 Çilli	0.199±0.001^{b}	0.937±0.001^{c}	1.191±0.010^{a}
Aygün	0.121±0.001^{c}	0.099±0.000^{d}	0.127±0.001^{e}

Figure 1. Changes in SOD activity of six *V. sativa* cultivars.



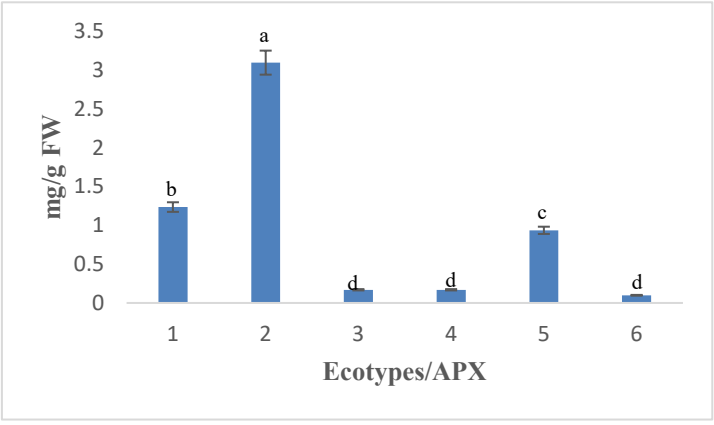
Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level b, c: SE; FW: Fresh weight (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Figure 2. Changes in Peroxidase activity of six *V. sativa* cultivars.



Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level a, b, c, d, e: SE; FW: Fresh weight (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Figure 3. Changes in APX activity of six *V. sativa* cultivars.



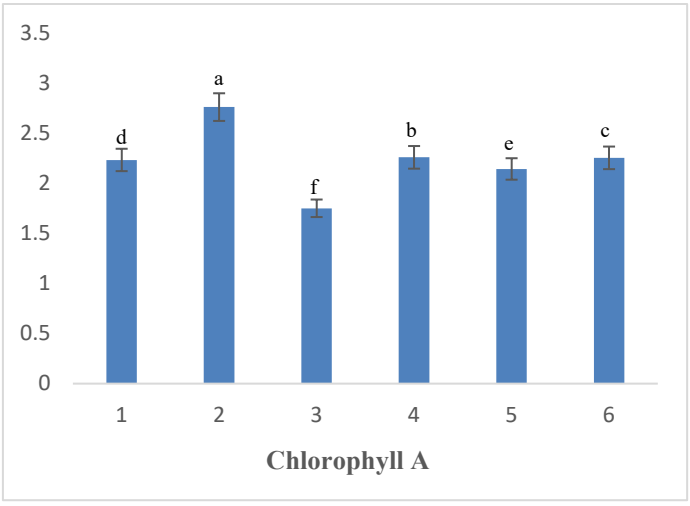
Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level a, b, c, d, e SE; FW: Fresh weight (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

The findings of the current report are in agreement with earlier studies reported in the literature. Increased APX, POX and SOD content at extreme cold conditions has been commonly reported in several crops, including pea (Theocharis *et al*., 2012; Anower *et al*., 2016). The effects of stress severity on antioxidant enzymes are proven to be linked to the stress tolerant ability (Cunningham *et al*., 2003; Couée *et al*., 2006). Our results indicated that under cold acclimation, a considerable increase in APX, SOD and POX activities was detected against oxidative stress in six faba cultivars. These findings are in agreement with those published by Tasgin *et al*. (2003) in the study on wheat germinating seedlings cold acclimizated at 0.01, 0.1 and 1mM salicyclic acid during 15 days. Their results demonstrate that acclimization could induce against cold stress and promote quickly freezing tolerance in winter wheat leaves by decreasing in freezing damage 41% 0.01, 22% by 0.1 and 19% by 1mM SA treatments. Chlorophyll content was different among tested cultivars at cold acclimation. Chlorophyll A content was observed with very different results compared to non acclimated. The highest chlorophyll A content was obtained in Tarım Beyazı (2.763 nmol g^{-1} FW), whereas the lowest chlorophyll A Ankara Moru (1. 752 nmol g^{-1} FW) at cold acclimation (Figure 4, Table 2). Similarly, the same cultivars observed a similar trend at the cold acclimation in Chlorophyll B (Figure 5, Table 2). In terms of carotenoid, the highest content was obtained in Tarım Beyazı (2.269 nmol g^{-1} FW) and Kansur (2.115 nmol g^{-1} FW), whereas the lowest carotenoid Ankara Moru (0.985 nmol g^{-1} FW) and 24 Çilli (1.181 nmol g^{-1} FW) at cold acclimation (Figure 6, Table 2).

Table 2. Changes in Chlorophyll A, Chlorophyll B and Carotenoid activity of six *V. sativa* cultivars.

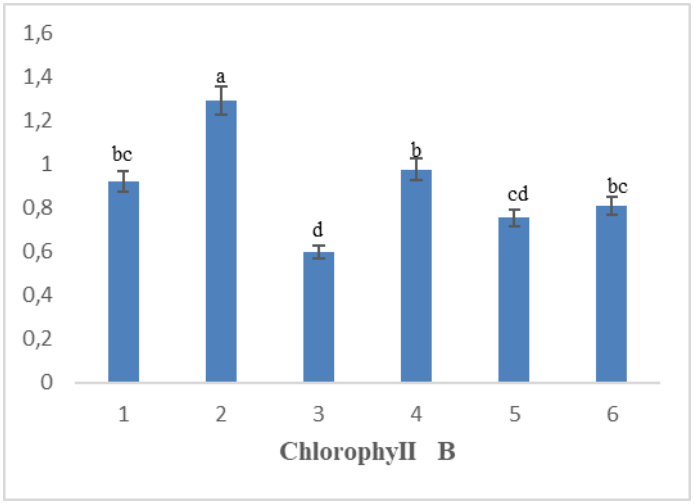
Ecotypes	Chlorophyll A	Chlorophyll B	Carotenoid
Doğu Beyazı	2.235±0.001^{d}	0.925±1.299^{bc}	1.910±0.010^{cd}
Tarım Beyazı	2.763±0.001^{a}	1.293±0.239^{a}	2.269±0.233^{a}
Ankara Moru	1.752±0.001^{f}	0.598±0.001^{d}	0.985±0.001^{e}
Kansur	2.261±0.001^{b}	0.978±0.001^{b}	2.115±0.001^{ab}
24 Çilli	2.2145±0.001^{e}	0.756±0.001^{cd}	1.181±0.001^{d}
Aygün	2.256±0.001^{c}	0.810±0.010^{bc}	2.038±0.001^{bc}

Figure 4. Changes in Chlorophyll A activity of six *V. sativa* cultivars.



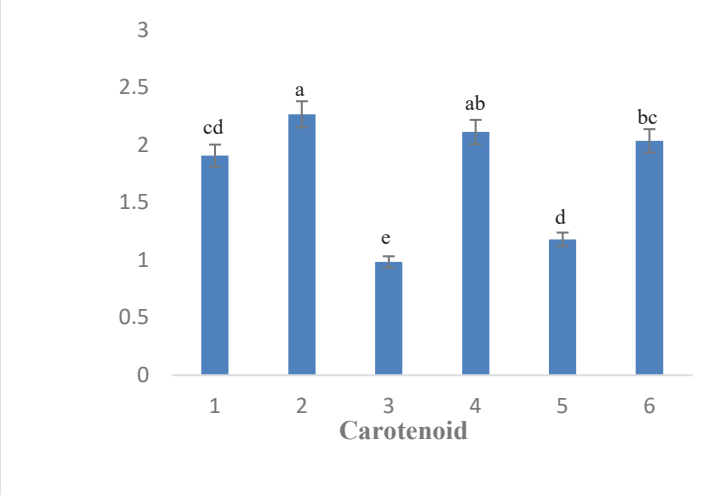
Differences between groups with different letters in a line are significant at the $P \leq 0.05$ significance level a, b, c, d, e SE. (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Figure 5. Changes in Chlorophyll B activity of six *V. sativa* cultivars.



Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level a, b, c, d, e SE. (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Figure 6. Changes in Carotenoid activity of six *V. sativa* cultivars.



Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level a, b, c, d, e SE (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Chlorophyll content is an efficient in vitro-selection way. However, it is significant to understand that more morphological and physiological stress factors must be explored for further screening and yield potential should be taken into account particularly for agricultural production. In our study, the highest chlorophyII A, B and carotenoid activity was observed in cold resistant cultivar Tarım Beyazı on 14 days, whereas, the lowest ones were assayed in cold tolerant Ankara Moru on 14 day at cold acclimate. However, a difference increase was detected in control cultivar. Moreover, chlorophyII content under non-acclimated and cold acclimation was statistically significant. This is in disagreement with an earlier report that indicated antioxidant activity is not linked to cold tolerance (Cansev *et al*., 2009). Siddique *et al*. (2018) indicated that Chl a and b values reduced the two cultivars (9 and 5) by 84% and 88% compared to controls. Carotenoid value also reduced in one cultivar (5) reduced by 54% compared to control. This outcome confirms that it could decrease the damage of antioxidant activity, at the

same time, the amounts of the chlorophyll enzyme have a direct role in the cold tolerance of *V. sativa* cultivars.

4. CONCLUSION

Cold acclimation protects plant tissues from injury under low temperature conditions and increases the productivity of crop species. Implement of cold acclimation in *V. sativa* is remarkably linked to the enrichment of antioxidant enzyme activity. *In vitro* selection methods implemented could be suggested for the cold resistance determination of seedlings in control conditions and important implications in selection breeding.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

KJ Senthil Kumar: Writing, Editing, Validation. **Ismail Bezirganoglu:** Experiment design, supervision. **Busra Yazicilar:** Laboratory work, statistical analysis. **Merve Simsek Geyik:** Laboratory work, statistical analysis. **Fatma Ozge Aslan:** Laboratory work, statistical analysis.

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The Studies on Seed Germination and *in Vitro* Cultures of *Salvia* L. Species from Turkish Flora

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Ex-situ conservation

Abstract

Turkey has a rich flora in terms of plant biodiversity, and economically important *Salvia* species because of medicinal properties are in danger of extinction. To prevent the extinction of *Salvia* species is important to solving the problems of seed germination and micropropagation. In this review, *in vitro* studies conducted on *Salvia* species from Turkish flora were listed by specifying danger categories. Seed germination studies carried out 18 *Salvia* species [*S. candidissima* Vahl., *S. cyanescens* Boiss. & Balansa (endemic/LR-lc), *S. dichroantha* Stapf (endemic/LR-lc), *S. fruticosa* Mill. (VU), S. *macrosiphon* Boiss., *S. microstegia* Boiss. et Bal., *S. nemorosa* L., *S. officinalis* L., *S. pomifera* ssp. *pomifera* L. (VU), *S. sclarea* L., *S. siirtica* Kahraman, Celep & Doğan sp. nov. (endemic/CR), *S. smyrnaea* Boiss. (endemic/EN), *S. tomentosa* Mill., *S. verbenaca* L., *S. verticillata* L., *S. virgata* Jacq., *S. viridis* L.]. *In vitro* culture studies were conducted on 16 *Salvia* species [*S. aethiopis* L., *S. albimaculata* Hedge and Huber-Morath (endemic/VU)-unsuccessful result, *S. cadmica* Boiss. (endemic/LR-lc), *S. candidissima* Vahl ssp. *occidentalis* Hedge, *S. chrysophylla* Stapf (endemic/LR-cd), *S. cryptantha* Montbret et Aucher ex Bentham (endemic/LR-lc), *S. euphratica* Montbret et Aucher ex. Bentham var. *euphratica* (endemic/LR-cd)-unsuccessful result, *S. fruticosa* (VU), *S. nemorosa, S. nydeggeri* Hub.-Mor.(endemic/EN)-unsuccessful result, *S. officinalis, S. sclarea, S. tomentosa, S. verticillata* ssp. *verticillata, S. virgata, S. viridis*]. In this review has been reported up to now *in vitro* cultures have been carried out 26 of 99 *Salvia* species, has clearly shows that new protocols should be established to protect endemic and threatened *Salvia* species.

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1. INTRODUCTION

Plant species in Turkey, which has a rich flora in terms of plant diversity, have been grouped in 145 families. Labiatae (syn. Lamiaceae) family, of which *Salvia* genus is a member, is one of the richest families in point of the number of species, especially the number of endemic species (İpek and Gürbüz, 2010). *Salvia* genus, which has about 1000 species in the world (Kahraman *et al*., 2011), has 99 species, 8 subspecies and 6 varieties (106 taxon) in Turkey (Tursun, 2019; Kayıkçı and Oğur, 2020). 56 of these species in Turkey is endemic (endemism percentage 56.5%) (İpek and Gürbüz, 2010) and the total number of endemic species are 57 with the *S. siirtica* described in 2011 (Kahraman *et al*., 2011).

Like other members of Lamiaceae family, *Salvia* species are also medicinal and aromatic plants, and rich in essential oils which have been used in food, perfumes, cosmetics and pharmaceuticals (Öğütçü *et al*., 2008; Tosun *et al*., 2009; Durgha *et al*., 2015). Many and varied useful secondary metabolites such as terpenoid, phenolic compounds, and essential oils have

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been isolated from the aerial parts of sage plants (Erişen *et al*., 2020). Essential oil compounds obtained from sage plants is a complex mixture of monoterpenes, sesquiterpenes and alcohols with their oxygen derivatives, aldehydes, esters, ethers, ketones, phenols and oxides (Elmas and Elmas, 2021). *Salvia* species contain many bioactive compounds that can be divided into mainly four groups: monoterpenes, diterpenes, triterpenes and phenolic components. The most common monoterpenes include: α- thujone and β-thujone, 1,8-cineole, and camphor; diterpenes include: carnosol, carnosic acid, rosmadial and manool; triterpenes include: oleanolic and ursolic acids. The phenolic components can be divided into two groups: phenolic acids (caffeic, vanillic, ferulic and rosmarinic acids) and flavonoids (luteolin, apigenin, and quercetin). Sesquiterpene α-humulene and viridiflorol are additionally present in the extracts of *Salvia* species (Jakovljević *et al.,* 2019). Especially, *S. officinalis* is one of the richest sources of antioxidants such as rosmarinic acid, caffeic acid, carnosic acid and oligomers of caffeic acid with multiple catechol groups (Dastanpoor *et al*., 2013). Various compounds from the different parts of *Salvia* plants are identified as over 160 polyphenols, comprising an array of phenolic acids and flavonoids (Lopresti, 2017). The gas chromatography-mass spectrometry (GC/MS) analysis of essential oil extracted from *S. officinalis* was reported to the presence of 49 components with camphor (25.14%), α- thujone (18.83%), 1,8-cineole (14.14%), viridiflorol (7.98%), β-thujone (4.46%) and β-caryophyllene (3.30%) as the major components (Sharma *et al*., 2019). *S. albimaculata, S. potentillifolia* Boiss. & Heldr. ex Bentham and *S. nydeggeri* from Soutwest Anatolia in Turkey were determine their antioxidant properties and phenolic compounds using the ultra performance liquid chromatography. Caffeic acid (3582.8 ± 2.5 µg/g, 2956.5 ± 4.6 µg/g and 2457.7 ± 3.1 µg/g) and 3,4-dihydroxy benzoic acid (1846.2 ± 3.1 µg/g, 2019.1 ± 2.2 µg/g and 1901.3 ± 1.5 µg/g) were found to be in the highest concentrations in *S. potentillifolia, S. albimaculata* and *S. nydeggeri*, respectively. Total amounts of phenolics and flavonoids were obtained highest in ethyl acetate extracts of samples. Antioxidant activity of *S. potentillifolia* was found to be higher than the other studied *Salvia* species (Kivrak *et al*., 2019).

So far published literature reported that the essential oils of *Salvia* species have showed the various pharmacological properties such as antioxidant, antibacterial, anti-neurodegenerative, anti-enzymatic (anticholinesterase, anti-urease, anti-tyrosinase, anti-elastase), antidiabetic and anti-tumour activities (Aşkun *et al*., 2010; Uysal *et al*., 2021). *Salvia* species growing in Turkey has been commonly consumed among the population as a diuretic, cold remedy, stomach relaxant, bloating remedy and tonic drink (Yilar *et al*., 2018). *Salvia* species in flora of Turkey have been used against various diseaes such as high fever, rheumatic pain, stomachache, peptic ulcer, urinary inflammations, catarrh, cold, wounds, flatulence, constipation, wards, sunstroke, and hemorrhage in Turkish folk medicine (Ustun *et al*., 2016; Dumanoğlu and Mokhtarzadeh, 2020).

Due to global warming, extreme drought, salinization, deforestation, erosion, human pressure, overgrazing, air-soil-water pollution, the unconscious over-collecting of economically valuable sage plants from nature is in danger of extinction this plant. Especially all of the endemic *Salvia* species are in the threatened category. 12 species of the genus *Salvia* (S. *anatolica* Hamzaoğlu & A. Duran, *S. ballsiana* (Rech.f.) Hedge, *S. freyniana* Bornm., *S. hedgeana* Dönmez, *S. marashica* A. İlçim, F. Celep & Doğan, *S. odontochlamys* Hedge, *S. pseudeuphratica* Rech., *S. quezelii* Hedge & Afzal-Rafii, *S. sericeo-tomentosa* Rech.f., *S. siirtica*, *S. tigrina* Hedge & Hub.-Mor., *S. vermifolia* Hedge & Hub.-Mor.) are critically endangered (CR) and 14 species (*S. adenophylla* Hedge & Hub.-Mor, *S. albimaculata*, *S. aytachii* Vural & N. Adıgüzel, *S. cedronella* Boiss., *S. cerino-pruinosa* Rech. var. *elazigensis* A.Karaman, F.Celep & Dogan, *S. cilicica* Boiss. & Kotschy, *S. ekimiana* F. Celep & Doğan sp. nova, *S. eriophora* Boiss. & Kotschy ex Boiss., *S. halophila* Hedge, *S. kronenburgii* Rech.f., *S. modesta* Boiss., *S. nydeggeri*, *S. smyrnaea*, *S. tobeyi* Hedge) are in endangered (EN) category.

Besides, since especially *S. fruticosa* (syn. *S. triloba*) and *S. cryptantha*, *S. multicaulis* Vahl, *S. sclarea* and *S. tomentosa* are also widely traded, they carry the risk of being endangered category (İpek and Gürbüz, 2010).

Conservation strategies include in-situ conservation, ex-situ conservation and cultivation studies. Within the scope of ex-situ conservation strategies, using plant biotechnology and *in vitro* techniques presents some important advantages, these techniques are in conservation of species that are especially under threatened of extinction and/or endemic (Bürün, 2021). "National Biologic Diversity Strategy and Action Plan" published in 2007 by Ministry of Environment and Forestry in Turkey also suggests to grow endangered species and produce them under control (Anonymous, 2007). To do this, it is necessary to collect seeds of that species first, then to establish seed germination protocol and determine the conditions for reproduction by seed as well as to multiply with other propagation techniques and do other culture studies. Therefore within ex-situ conservation strategies, propagation studies of natural species has been important in terms of protection of genetic diversity. *In vitro* techniques are being used in secondary metabolite production and breeding besides conservation and propagation of *Salvia* species which is a valuable genus from the medicinal and aromatic characteristics (Surgun-Acar and Bürün, 2017). *In vitro* culture of medicinal *Salvia* species for the purpose of extraction of bioactive constituents may face certain limitations such as climate, season, water availability, various environmental stresses, diseases, pests and shortage of naturally growing plants (Ghanbar *et al*., 2016). These difficulties and challenges such as the differences in terrain and climate conditions, low metabolite yield and quality and more labor needed a new biotechnological approaches in order to enable more economic, higher metabolite yield and quality compared to the conventional methods (Demirci *et al*., 2015). Therefore, *in vitro* cell and organ culture techniques have emerged as an alternative way to plant growing under field conditions for secondary metabolite production (Çalışkan *et al*., 2019).

An important priority in efforts to conserve rare species is their seed germination. Because germination and seedling emergence are the most critical phases in the plant life cycle (Javaid *et al*., 2018). However, the main global problem of *Salvia* species has been the germination of their seeds and so far an integral solution has not been reported for this (Abdollahi *et al*., 2012a; Abdollahi *et al*., 2012b). The aim of this review article is to review the seed germination and *in vitro* culture studies of the economically important *Salvia* genus in Turkey and to evaluate them in terms of ex-situ conservation strategies.

1.2. Seed Germination Studies on *Salvia* Species in Flora of Turkey

The physiological events related to seed germination are the first step of plant life (Tursun, 2019). Seed germination studies of rare, endangered and endemic species have a great importance in determining in situ and ex situ conservation strategies. Seed germination is the only way to preserve genetic diversity, and many plant species are at risk of being extinct. Therefore, investigations on the germination abilities of endangered plant seeds are important to conservation for the next generation (Subaşı and Güvensen, 2010).

Seed germination studies of *Salvia* species has been carried out only in 18 species (4 of which is endemic) and the first has been reported in *S. fruticosa* by Thanos and Doussi (1995). *Salvia* genus has 99 species in Turkish flora. There are 12 species in critically endangered (CR), 14 species in endangered (EN), 15 species in vulnerable (VU), 17 species in the least concern (LR-lc), 2 species in near threatened (LR-nt) categories, and 1 species (*S. reeseana* Hedge & Hub.-Mor.) is data deficient (DD). Germination percentages has been found between 21.25% (*S. siirtica*) and 97% (*S. verbenaca*) in 18 species studied, including 1 species (*S. siirtica*) in endemic/CR, 2 species (*S. fruticosa*, *S. pomifera* L. ssp. *pomifera*) in VU, 1 species (*S. smyrnaea*) in endemic/EN, 2 species (*S. cyanescens, S. dichroantha*) endemic/LR-lc and another 12 species not endangered categories. As it is seen in Table 1, few studies have been

conducted out on seed germination, and seed germination has been investigated only in one (*S. siirtica*) of 12 species of which is in the CR category. Seed germination has been studied only on *S. smyrnaea* from 13 species in the EN category, and 66.6% germination has been achieved with some applications.

Table 1. Seed germination studies in *Salvia* species in flora of Turkey (The species are listed in an alphabetic order).

Species	Endemic/ Danger status	Germination conditions and/or Pre-treatments	Germination percentage	Reference
S. candidissima	–/–*	Gibberellic acid (0, 250, 500, 750 mg l^{-1}) and temperature (5, 10, 15, 20, 25, 30 and 35°C) treatments	4.4%	(Dadaşoğlu and Özer, 2014)
S. cyanescens	+/LR(lc)*	Pretreated for 5 minutes at -5°C / top of paper at 25°C	78%	(Yücel and Yilmaz, 2009)
S. dichroantha	+/LR(lc)*	In darkness, at 20°C	80%	(Thanos and Doussi, 1995)
		Storage at 4°C for 10 days, then soaking in 500 ppm Gibberellin solution	50.2%	(Özcan *et al*., 2014)
		Polymer coating+GA_3 Application GA_3 at 25°C	85% 82%	(Sönmez *et al*., 2019)
S. macrosiphon	–/–*	Gibberellic acid [0 (control), 100, 150 and 200 mg l^{-1}]	62%	(Abdollahi *et al*., 2012a)
		Gibberellic acid(150 mg l^{-1}) under drought stress (PEG)	significant correlation between germination and drought	(Abdollahi *et al*., 2012b)
S. microstegia	–/–*	Gibberellic acid (0, 250, 500, 750 mg l^{-1}) and temperature (5, 10, 15, 20, 25, 30 and 35°C) treatments	50%	(Dadaşoğlu and Özer, 2014)
S. nemorosa	–/–*	Gibberellic acid (0, 250, 500, 750 mg l^{-1}) and temperature (5, 10, 15, 20, 25, 30 and 35°C) treatments	50%	(Dadaşoğlu and Özer, 2014)
S. officinalis	–/–*	Seed priming (10, 20, 30°C for 0, 12, 24, 48 h.)	77.5%	(Dastanpoor *et al*., 2013)
		Mercury (II) chloride (0.1%) for 5, 10 minutes or sodium hypochlorite (30%) for 10, 15 minutes	50%	(Yaschurevskaya and Cherednichenko, 2017)
S. pomifera ssp. *pomifera*	–/VU*	In darkness, at 15°C	70%	(Thanos and Doussi, 1995)
		At 25°C	42%	(Joshi and Pant, 2010)
S. pomifera		In MS medium containing 80 ppm chitosan and pH:7-8	85.8%-86.7%	(Ghanbar *et al*., 2012)

		Storage at 4°C for 10 days, then soaking in 500 ppm Gibberellin solution	47.6%	(Özcan *et al.,* 2014)
S. sclarea	–/–*	In paper at 2 days open in room temparature and then incubated at 20°C under continuous dark	89.3%	(Kumar and Sharma, 2012)
		Mercury (II) chloride (0.1%) for 5, 10 minutes or sodium hypochlorite (30%) for 10, 15 minutes	90%	(Yaschurevskaya and Cherednichenko, 2017)
S. siirtica sp. nov.	+/CR**	Stratification (28 day in fine sand maintaining in refrigerator of 4°C)	21.2%	(Arslan *et al*., 2017)
S. smyrnaea	+/EN*	Stratification (at 5°C, 45 days), in dark (25/15°C), 250 ppm GA_3 application	66.6%	(Subaşı and Güvensen, 2010)
S. tomentosa	–/–*	Storage at 4°C for 10 days, then soaking in 500 ppm Gibberellin solution	39.6%	(Özcan *et al*., 2014)
S. verbenaca	–/–*	Scabbing treatment	53%	(Khakpor *et al*., 2011)
		12 hours light/12 hours dark; the complete darkness regime of 24 hours dark; darkness and day/night temperatures: 30/20˚C; 20/15˚C; 25/12˚C	97%	(Javaid *et al*., 2018)
S. verticillata	–/–*	Gibberellic acid (0, 250, 500, 750 mg l^{-1}) and temperature (5, 10, 15, 20, 25, 30 and 35°C) treatments	32.3%	(Dadaşoğlu and Özer, 2014)
		Giberellic acid (2000 ppm) in dark at 26/16°C	74%	(Tursun, 2019)
		At -80°C for 4 days, in dark at 20±1°C	57-67%	(Tursun, 2020)
S. virgata	–/–*	Gibberellic acid (0, 250, 500, 750 mg l^{-1}) and temperature (5, 10, 15, 20, 25, 30 and 35°C) treatments	44%	(Dadaşoğlu and Özer, 2014)
S. viridis	–/–*	Mercury (II) chloride (0.1%) for 5, 10 minutes or sodium hypochlorite (30%) for 10, 15 minutes	90%	(Yaschurevskaya and Cherednichenko, 2017)

*(İpek and Gürbüz, 2010), **(Kahraman *et al*., 2011). IUCN: The International Union for Conservation of Nature. IUCN Categories: CR-Critically Endangered, EN-Endangered, VU-Vulnarable, LR-Lower Risk LR(cd)-Conservation Dependent, LR(lc)-Least Concern. MS: Murashige-Skoog (1962)

1.3. *In vitro* Cultures on *Salvia* Species in Flora of Turkey

Plant biotechnology allows efficient, fast, large scale and easily production of plant materials and their isolated compounds. *In vitro* cultures offer many advantages such as independence from climatic and geographical conditions, the possibility of efficient propagation of species in a short time from a small quantity of explants as a primary material (Grzegorczyk-Karolak *et al*., 2021). *Salvia* species is propagated different parts such as shoot tip, nodal segment, leaf, hypocotyl of the sterile plants via seeds germination. In nature, after

Salvia seeds remain dormant for a long time, they germinate very slowly. Therefore, *in vitro* cultures for large scale multiplication would be a viable option, and several medicinal plants are considered an effective tool to multiply difficult to propagate, useful, rare and endangered species for different commercial purposes (Jafari *et al*., 2017a).

Table 2 shows that *in vitro* culture applications on *Salvia* species from Turkish flora were investigated after the 1999s. *In vitro* culture studies have been encountered only in 16 species of the ones that exist in Turkey and successful results have been obtained in 13 species. Mostly studied species are *S. sclarea* and *S. fruticosa. S. sclarea* is not an endemic or threatened with extinction species but it is heavily traded. *S. fruticosa,* on the other hand, is a commercially traded species in the VU category. While there is only eight study for secondary metabolite production of *S. cryptantha* (endemic/LR-Ic) and *S. tomentosa*, which are traded, there is not an *in vitro* culture study on *S. multicaulis* (Table 2).

Table 2. Seed germination studies in *Salvia* species in flora of Turkey (The species are listed in an alphabetic order).

Species	Endemic / Threatened position*	Explant	Medium	Goal/Result	Reference
S. aethiopis	–/–	Seed	MS	Callus induction for utilization in secondary metabolite production	(Rezaeieh *et al*., 2012)
		Hypocotyls	MS	Production of secondary metabolites	(Vulganová *et al*., 2019)
S. albimaculata	+/EN	Nodal explant and shoot tip	MS	Micropropagation/ unsuccessful	(Uyanık, 2017)
S. cadmica	+/LR(lc)	Seed	MS	Callus induction for utilization in secondary metabolite production	(Rezaeieh *et al*., 2012)
S. candidissima ssp. *occidentalis*	–/–	Seed	MS	Callus induction for utilization in secondary metabolite production	(Rezaeieh *et al*., 2012)
S. chrysophylla	+/VU	Nodal explant and shoot tip	MS	Micropropagation/ regeneration, rooting and acclimatizion were achieved	(Uyanık, 2017)
S. cryptantha	+/LR(lc)	Seed	MS	Callus induction for utilization in secondary metabolite production	(Rezaeieh *et al*., 2012)
S. euphratica var. e*uphratica*	+/LR(lc)	Nodal explant and shoot tip	MS	Micropropagation/ unseccessful	(Uyanık, 2017)
S. fruticosa	–/VU	Leaf (from young plants)	MS	Somatic embryogenesis and rosmarinic acid accumulation	(Kintzios *et al*., 1999)
		Shoot tips (from *in vivo* and *in vitro* seedlings)	MS and B_5	Callus, cell suspension and root cultures, rosmarinic acid accumulation	(Karam *et al*., 2003)

		Shoot tips (from *in vitro* seedlings)	MS, NN, B_5	Micropropagation and accumulation of essential oils/A protocol was developed for micropropagation and essential oil in microshoots was high	(Arikat *et al*., 2004)
		Cell cultures obtained leaf	LS	The production variability of different metabolites (phenols and sterols) from cell suspension culture	(Kümmritz *et al*., 2014)
S. nemorosa	–/–	Shoot tip, leaf lamina and petiol	MS	Micropropagation/ Protocol described	(Skała and Wysokińska, 2004)
S. nydeggeri	+/EN	Nodal explant and shoot tip	MS	Micropropagation/ unseccessful	(Uyanık, 2017)
S. officinalis	–/–	Leaf explants	MS	Regeneration, micropropagation, callus cultures and somatic embryogenesis	(Ioja-Boldura *et al*., 2010)
		Shoot tip	MS	Synthetic seed production	(Grzegorczyk and Wysokińska, 2011)
		Cell cultures obtained leaf	LS	The production variability of different metabolites (phenols and sterols) from cell suspension culture	(Kümmritz *et al*., 2014)
		Nodal segments	MS	Efficient method for micropropagation to evaluate flavonoid content and antioxidant capacity	(Petrova *et al*., 2015)
		Leaf and internode	MS	Influence of putrescine and thidiazuron on *in vitro* organogenesis	(Jafari *et al*., 2017a)
		Leaf and internode	MS	Indirect organogenesis and plant regeneration	(Jafari *et al*., 2017b)
S. sclarea	–/–	Immature zygotic embryo and cotyledons	MS	Organogenesis/ Establish a plant regeneration system	(Liu *et al*., 2000)
		Leaf (35-day old seedlings)	MS	Investigate the process of transformation/*In vitro* induction of crown galls	(Khawar *et al*., 2003)
		Axillary and apical buds	MS	Micropropagation	(Mascarello *et al*., 2006)

		(from adult plants)			
		Nodal segments	MS	Synthetic seed production	(Durgha *et al*., 2015)
		Shoot tips and axillary node (from16-day-old seedlings)	MS	Micropropagation and conservation/ this work describes successful regeneration	(Ghanbar *et al*., 2016)
		Hypocotyls	MS	Production of secondary metabolites	(Vulganová *et al*., 2019)
		Nodal segments	MS	Micropropagation/ Protocol described	(Erişen *et al*., 2020)
		Leaves and/or callus	MS	to investigate chemical properties and biological activities	(Saruhan-Fidan *et al*., 2021)
S. tomentosa	–/–	Seed	MS	Callus induction for utilization in secondary metabolite production	(Rezaeieh *et al*., 2012)
S. verticillata ssp. *verticillata*	–/–	Seed	MS	Callus induction for utilization in secondary metabolite production	(Rezaeieh *et al*., 2012)
S. virgata	–/–	Cell cultures obtained leaf	LS	The production variability of different metabolites (phenols and sterols) from cell suspension culture	(Kümmritz *et al*., 2014)
		Nodal explants	MS	Phenolic acids production in shoot cultures	(Dowom *et al*., 2017)
S. viridis	–/–	Hairy root cultures	MS	Production of polyphenolic compounds	(Grzegorczyk-Karolak *et al*., 2018)
		Shoot and/or callus cultures	–	to investigate their potential for the accumulation of phenolic compounds	(Grzegorczyk-Karolak *et al*., 2019)
		In vitro shoot culture	MS	to investigate the effects of purine-type cytokinins on the polyphenolic compounds accumulation	(Grzegorczyk-Karolak *et al*., 2020)
		Shoot	MS	Synthetic seed production	(Grzegorczyk-Karolak *et al*., 2021)

*(İpek and Gürbüz, 2010). MS: (Murashige and Skoog, 1962), NN: (Nitsch and Nitsch, 1969), B_5: Gamborg's medium, LS: Linsmaier and Skoog.

The majority of *in vitro* studies on *Salvia* species are micropropagation, organogenesis, plant regeneration, callus induction, secondary metabolite production and synthetic seed production. The micropropagation of *S. albimaculata* and *S. nydeggeri* which is endemic in EN category has been studied; however these efforts have not been successful. Furthermore, successful results can not also be obtained from the study on micropropagation of *S. euphratica* var. *euphratica* which is endemic in LR-lc category (Table 2).

4. CONCLUSION

Turkey exhibits great wealth in terms of plant species number, biodiversity and endemism as a result of different geographical factors such as climate, altitude, soil structure. The genus *Salvia*, which has a high endemism rate, has medicinal and aromatic properties such as antibacterial, antifungal, antiviral, antiseptic, analgesic, antidiabetic. However, many *Salvia* species are under threat of extinction because of unconscious, uncontrolled and excessive collection. It is essential to implement appropriate ex-situ strategies to conserve and grow especially endemic or threatened species. In ex-situ conservation strategies will be managed according to the information about the species, thus success in ex-situ conservation will be achieved. For this reason, seed germination conditions of the species should be determined first and propagation by seed should be investigated. In addition, for species that cannot be produced by seeds or even if they can be propagated by seeds, their production by *in vitro* techniques should be investigated and appropriate micropropagation protocols should be established. In order to protect the plant diversity of Turkish flora, it is essential to carry out these studies and to implement conservation strategies. It is important to use plant biotechnology approaches, especially *in vitro* cultures, in the conservation of threatened plant species. Some conservation strategies for endangered and traded *Salvia* species in Turkey must be applied immediately. Only 26 species out of 99 species have been established on seed germination and *in vitro* culture studies. Compared to the total number of *Salvia* species found in Turkey, the rate of species studied *in vitro* is very low, at 26%. Conservation strategies need to focus on unstudied species and determine appropriate protocols urgently.

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